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<b>(21) International Application Number:</b> PCT/US97/15941 <b>(22) International Filing Date:</b> 9 September 1997 (09.09.97)  <b>(30) Priority Data:</b> 08/814,877 9 September 1996 (09.09.96) US 08/850,880 2 May 1997 (02.05.97) US  <b>(71) Applicant:</b> WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US).  <b>(72) Inventors:</b> REZNIKOFF, William, S.; 733 Lakewood Boulevard, Maple Bluff, WI 53704 (US). GORYSHIN, Igor, Yu; Apartment D, 23 University Houses, Madison, WI 53705 (US). ZHOU, Hong; Apartment 104, 5331 Brody Drive, Madison, WI 53705 (US).  <b>(74) Agent:</b> BERSON, Bennett, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> SYSTEM FOR <i>IN VITRO</i> TRANSPOSITION USING MODIFIED Tn5 TRANSPOSASE  <b>(57) Abstract</b> <p>A system for <i>in vitro</i> transposition includes a donor DNA that includes a transposable element flanked by a pair of bacterial transposon Tn5 outside end repeat sequences, a target DNA into which the transposable element can transpose, and a modified Tn5 transposase having higher binding avidity to the outside end repeat sequences and being less likely to assume an inactive multimer form than wild type Tn5 transposase.</p>		

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SYSTEM FOR *IN VITRO* TRANSPOSITION USING MODIFIED TNS TRANSPOSASE

## CROSS-REFERENCE TO RELATED APPLICATION

This patent application is a continuation-in-part of a patent application entitled "System for *In Vitro* Transposition," filed March 11, 1997, for which no serial number has yet been accorded. Applicants have petitioned for a filing date of September 9, 1996 to be accorded to the parent application.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT  
Not applicable.

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## BACKGROUND OF THE INVENTION

The present invention relates generally to the field of transposable nucleic acid and, more particularly to production and use of a modified transposase enzyme in a system for introducing genetic changes to nucleic acid.

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Transposable genetic elements are DNA sequences, found in a wide variety of prokaryotic and eukaryotic organisms, that can move or transpose from one position to another position in a genome. *In vivo*, intra-chromosomal transpositions as well as transpositions between chromosomal and non-chromosomal genetic material are known. In several systems, transposition is known to be under the control of a transposase enzyme that is typically encoded by the transposable element. The genetic structures and transposition mechanisms of various transposable elements are summarized, for example, in "Transposable Genetic Elements" in "The Encyclopedia of Molecular Biology," Kendrew and Lawrence, Eds., Blackwell Science, Ltd., Oxford (1994), incorporated herein by reference.

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*In vitro* transposition systems that utilize the particular transposable elements of bacteriophage Mu and bacterial transposon Tn10 have been described, by the research groups of

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5 Kiyoshi Mizuuchi and Nancy Kleckner, respectively.

10 The bacteriophage Mu system was first described by Mizuuchi, K., "In Vitro Transposition of Bacteria Phage Mu: A Biochemical Approach to a Novel Replication Reaction," Cell:785-794 (1983) and Craigie, R. et al., "A Defined System for the DNA Strand-Transfer Reaction at the Initiation of Bacteriophage Mu Transposition: Protein and DNA Substrate Requirements," P.N.A.S. U.S.A. 82:7570-7574 (1985). The DNA donor substrate (mini-Mu) for Mu in vitro reaction normally requires six Mu transposase binding sites (three of about 30 bp at each end) and an enhancer sequence located about 1 kb from the left end. The donor plasmid must be supercoiled. Proteins required are Mu-encoded A and B proteins and host-encoded HU and IHF proteins. Lavoie, B.D, and G. Chaconas, "Transposition of phage Mu DNA," Curr. Topics Microbiol. Immunol. 204:83-99 (1995). The Mu-based system is disfavored for in vitro transposition system applications because the Mu termini are complex and sophisticated and because transposition requires additional proteins above and beyond the transposase.

25 The Tn10 system was described by Morisato, D. and N. Kleckner, "Tn10 Transposition and Circle Formation in vitro," Cell 51:101-111 (1987) and by Benjamin, H. W. and N. Kleckner, "Excision Of Tn10 from the Donor Site During Transposition Occurs By Flush Double-Strand Cleavages at the Transposon Termini," P.N.A.S. U.S.A. 89:4648-4652 (1992). The Tn10 system involves the a supercoiled circular DNA molecule carrying the transposable element (or a linear DNA molecule plus E. coli IHF protein). The transposable element is defined by complex 42 bp terminal sequences with IHF binding site adjacent to the inverted repeat. In fact, even longer (81 bp) ends of Tn10 were used in reported experiments. Sakai, J. et al., "Identification and Characterization of Pre-Cleavage Synaptic Complex that is an Early Intermediate in Tn10 transposition," E.M.B.O. J. 14:4374-4383 (1995). In the Tn10 system, chemical treatment of the transposase protein is essential to support active transposition. In addition, the termini of the Tn10 element limit its utility in a generalized in vitro

5 transposition system.

Both the Mu- and Tn10-based *in vitro* transposition systems are further limited in that they are active only on covalently closed circular, supercoiled DNA targets. What is desired is a more broadly applicable *in vitro* transposition system that  
10 utilizes shorter, more well defined termini and which is active on target DNA of any structure (linear, relaxed circular, and supercoiled circular DNA).

#### BRIEF SUMMARY OF THE INVENTION

The present invention is summarized in that an *in vitro*  
15 transposition system comprises a preparation of a suitably modified transposase of bacterial transposon Tn5, a donor DNA molecule that includes a transposable element, a target DNA molecule into which the transposable element can transpose, all provided in a suitable reaction buffer.

20 The transposable element of the donor DNA molecule is characterized as a transposable DNA sequence of interest, the DNA sequence of interest being flanked at its 5'- and 3'-ends by short repeat sequences that are acted upon *in trans* by Tn5 transposase.

25 The invention is further summarized in that the suitably modified transposase enzyme comprises two classes of differences from wild type Tn5 transposase, where each class has a separate measurable effect upon the overall transposition activity of the enzyme and where a greater effect is observed  
30 when both modifications are present. The suitably modified enzyme both (1) binds to the repeat sequences of the donor DNA with greater avidity than wild type Tn5 transposase ("class (1) mutation") and (2) is less likely than the wild type protein to assume an inactive multimeric form ("class (2) mutation"). A  
35 suitably modified Tn5 transposase of the present invention that contains both class (1) and class (2) modifications induces at least about 100-fold ( $\pm 10\%$ ) more transposition than the wild type enzyme, when tested in combination in an *in vivo* conjugation assay as described by Weinreich, M.D., "Evidence  
40 that the *cis* Preference of the Tn5 Transposase is Caused by

5 Nonproductive Multimerization," Genes and Development 8:2363-  
2374 (1994), incorporated herein by reference. Under optimal  
conditions, transposition using the modified transposase may be  
higher. A modified transposase containing only a class (1)  
10 mutation binds to the repeat sequences with sufficiently  
greater avidity than the wild type Tn5 transposase that such a  
Tn5 transposase induces about 5- to 50-fold more transposition  
than the wild type enzyme, when measured in vivo. A modified  
transposase containing only a class (2) mutation is  
15 sufficiently less likely than the wild type Tn5 transposase to  
assume the multimeric form that such a Tn5 transposase also  
induces about 5- to 50-fold more transposition than the wild  
type enzyme, when measured in vivo.

In another aspect, the invention is summarized in that a  
method for transposing the transposable element from the donor  
20 DNA into the target DNA in vitro includes the steps of mixing  
together the suitably modified Tn5 transposase protein, the  
donor DNA, and the target DNA in a suitable reaction buffer,  
allowing the enzyme to bind to the flanking repeat sequences of  
the donor DNA at a temperature greater than 0°C, but no higher  
25 than about 28°C, and then raising the temperature to  
physiological temperature (about 37°C) whereupon cleavage and  
strand transfer can occur.

It is an object of the present invention to provide a  
useful in vitro transposition system having few structural  
30 requirements and high efficiency.

It is another object of the present invention to provide a  
method that can be broadly applied in various ways, such as to  
create absolute defective mutants, to provide selective markers  
to target DNA, to provide portable regions of homology to a  
35 target DNA, to facilitate insertion of specialized DNA  
sequences into target DNA, to provide primer binding sites or  
tags for DNA sequencing, to facilitate production of genetic  
fusions for gene expression studies and protein domain mapping,  
as well as to bring together other desired combinations of DNA  
40 sequences (combinatorial genetics).

It is a feature of the present invention that the modified

5 transposase enzyme binds more tightly to DNA than does wild type Tn5 transposase.

It is an advantage of the present invention that the modified transposase facilitates *in vitro* transposition reaction rates of at least about 100-fold higher than can be achieved using wild type transposase (as measured *in vivo*). It is noted that the wild-type Tn5 transposase shows no detectable *in vitro* activity in the system of the present invention. Thus, while it is difficult to calculate an upper limit to the increase in activity, it is clear that hundreds, if not thousands, of colonies are observed when the products of *in vitro* transposition are assayed *in vivo*.

It is another advantage of the present invention that *in vitro* transposition using this system can utilize donor DNA and target DNA that is circular or linear.

20 It is yet another advantage of the present invention that *in vitro* transposition using this system requires no outside high energy source and no other protein other than the modified transposase.

Other objects, features, and advantages of the present invention will become apparent upon consideration of the following detailed description.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 depicts test plasmid pRZTL1, used herein to demonstrate transposition *in vitro* of a transposable element located between a pair of Tn5 outside end termini. Plasmid pRZTL1 is also shown and described in SEQ ID NO:3.

Fig. 2 depicts an electrophoretic analysis of plasmid pRZTL1 before and after *in vitro* transposition. Data obtained using both circular and linear plasmid substrates are shown.

35 Fig. 3 is an electrophoretic analysis of plasmid pRZTL1 after *in vitro* transposition, including further analysis of the molecular species obtained using circular and linear plasmid substrates.

Fig. 4 shows plasmids pRZ1496, pRZ5451 and pRZTL1, which are detailed in the specification.

5 Fig. 5 shows a plot of papillae per colony over time for various mutant OE sequences tested in vivo against EK54/MA56 transposase.

Fig. 6 shows a plot of papillae per colony over time for various mutant OE sequences with a smaller Y-axis than is shown  
10 in Fig. 5 tested against EK54/MA56 transposase.

Fig. 7 shows a plot of papillae per colony over time for various mutant OE sequences tested against MA56 Tn5 transposase.

Fig. 8 shows in vivo transposition using two preferred  
15 mutants, tested against MA56 and EK54/MA56 transposase.

#### DETAILED DESCRIPTION OF THE INVENTION

It will be appreciated that this technique provides a simple, in vitro system for introducing any transposable element from a donor DNA into a target DNA. It is generally  
20 accepted and understood that Tn5 transposition requires only a pair of OE termini, located to either side of the transposable element. These OE termini are generally thought to be 18 or 19 bases in length and are inverted repeats relative to one another. Johnson, R. C., and W. S. Reznikoff, Nature 304:280  
25 (1983), incorporated herein by reference. The Tn5 inverted repeat sequences, which are referred to as "termini" even though they need not be at the termini of the donor DNA molecule, are well known and understood.

Apart from the need to flank the desired transposable element with standard Tn5 outside end ("OE") termini, few other  
30 requirements on either the donor DNA or the target DNA are envisioned. It is thought that Tn5 has few, if any, preferences for insertion sites, so it is possible to use the system to introduce desired sequences at random into target  
35 DNA. Therefore, it is believed that this method, employing the modified transposase described herein and a simple donor DNA, is broadly applicable to introduce changes into any target DNA, without regard to its nucleotide sequence. It will, thus, be applied to many problems of interest to those skilled in the  
40 art of molecular biology.



5 In the method, the modified transposase protein is combined in a suitable reaction buffer with the donor DNA and the target DNA. A suitable reaction buffer permits the transposition reaction to occur. A preferred, but not necessarily optimized, buffer contains spermidine to condense  
10 the DNA, glutamate, and magnesium, as well as a detergent, which is preferably 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate ("CHAPS"). The mixture can be incubated at a temperature greater than 0°C and as high as about 28°C to facilitate binding of the enzyme to the OE termini. Under the  
15 buffer conditions used by the inventors in the Examples, a pretreatment temperature of 30°C was not adequate. A preferred temperature range is between 16°C and 28°C. A most preferred pretreatment temperature is about 20°C. Under different buffer conditions, however, it may be possible to use other below-  
20 physiological temperatures for the binding step. After a short pretreatment period of time (which has not been optimized, but which may be as little as 30 minutes or as much as 2 hours, and is typically 1 hour), the reaction mixture is diluted with 2 volumes of a suitable reaction buffer and shifted to  
25 physiological conditions for several more hours (say 2-3 hours) to permit cleavage and strand transfer to occur. A temperature of 37°C, or thereabouts, is adequate. After about 3 hours, the rate of transposition decreases markedly. The reaction can be stopped by phenol-chloroform extraction and can then be  
30 desalted by ethanol precipitation.

When the DNA has been purified using conventional purification tools, it is possible to employ simpler reaction conditions in the *in vitro* transposition method. DNA of sufficiently high purity can be prepared by passing the DNA  
35 preparation through a resin of the type now commonly used in the molecular biology laboratory, such as the Qiagen resin of the Qiagen plasmid purification kit (Catalog No. 12162). When such higher quality DNA is employed, CHAPS can be omitted from the reaction buffer. When CHAPS is eliminated from the  
40 reaction buffer, the reactants need not be diluted in the manner described above. Also, the low temperature incubation

5 step noted above can be eliminated in favor of a single incubation for cleavage and strand transfer at physiological conditions. A three hour incubation at 37°C is sufficient.

10 Following the reaction and subsequent extraction steps, transposition can be assayed by introducing the nucleic acid reaction products into suitable bacterial host cells (e.g., *E. coli* K-12 DH5α cells (recA<sup>-</sup>); commercially available from Life Technologies (Gibco-BRL)) preferably by electroporation, described by Dower et al., Nuc. Acids. Res. 16:6127 (1988), and monitoring for evidence of transposition, as is described  
15 elsewhere herein.

Those persons skilled in the art will appreciate that apart from the changes noted herein, the transposition reaction can proceed under much the same conditions as would be found in an *in vivo* reaction. Yet, the modified transposase described  
20 herein so increases the level of transposition activity that it is now possible to carry out this reaction *in vitro* where this has not previously been possible. The rates of reaction are even greater when the modified transposase is coupled with an optimized buffer and temperature conditions noted herein.

25 In another aspect, the present invention is a preparation of a modified Tn5 transposase enzyme that differs from wild type Tn5 transposase in that it (1) binds to the repeat sequences of the donor DNA with greater avidity than wild type Tn5 transposase and (2) is less likely than the wild type  
30 protein to assume an inactive multimeric form. An enzyme having these requirements can be obtained from a bacterial host cell containing an expressible gene for the modified enzyme that is under the control of a promoter active in the host cell. Genetic material that encodes the modified Tn5  
35 transposase can be introduced (e.g., by electroporation) into suitable bacterial host cells capable of supporting expression of the genetic material. Known methods for overproducing and preparing other Tn5 transposase mutants are suitably employed. For example, Weinreich, M. D., et al., *supra*, describes a  
40 suitable method for overproducing a Tn5 transposase. A second method for purifying Tn5 transposase was described in de la

5 Cruz, N. B., et al., "Characterization of the Tn5 Transposase  
and Inhibitor Proteins: A Model for the Inhibition of  
Transposition," J. Bact. 175:6932-6938 (1993), also  
incorporated herein by reference. It is noted that induction  
can be carried out at temperatures below 37°C, which is the  
10 temperature used by de la Cruz, et al. Temperatures at least in  
the range of 33 to 37°C are suitable. The inventors have  
determined that the method for preparing the modified  
transposase of the present invention is not critical to success  
of the method, as various preparation strategies have been used  
15 with equal success.

Alternatively, the protein can be chemically synthesized,  
in a manner known to the art, using the amino acid sequence  
attached hereto as SEQ ID NO:2 as a guide. It is also possible  
to prepare a genetic construct that encodes the modified  
20 protein (and associated transcription and translation signals)  
by using standard recombinant DNA methods familiar to molecular  
biologists. The genetic material useful for preparing such  
constructs can be obtained from existing Tn5 constructs, or can  
be prepared using known methods for introducing mutations into  
25 genetic material (e.g., random mutagenesis PCR or site-directed  
mutagenesis) or some combination of both methods. The genetic  
sequence that encodes the protein shown in SEQ ID NO:2 is set  
forth in SEQ ID NO:1.

The nucleic acid and amino acid sequence of wild type Tn5  
30 transposase are known and published. N.C.B.I. Accession Number  
U00004 L19385, incorporated herein by reference.

In a preferred embodiment, the improved avidity of the  
modified transposase for the repeat sequences for OE termini  
(class (1) mutation) can be achieved by providing a lysine  
35 residue at amino acid 54, which is glutamic acid in wild type  
Tn5 transposase. The mutation strongly alters the preference  
of the transposase for OE termini, as opposed to inside end  
("IE") termini. The higher binding of this mutation, known as  
EK54, to OE termini results in a transposition rate that is  
40 about 10-fold higher than is seen with wild type transposase.  
A similar change at position 54 to valine (mutant EV54) also

5 results in somewhat increased binding/transposition for OE  
termini, as does a threonine-to-proline change at position 47  
(mutant TP47; about 10-fold higher). It is believed that  
other, comparable transposase mutations (in one or more amino  
acids) that increase binding avidity for OE termini may also be  
10 obtained which would function as well or better in the *in vitro*  
assay described herein.

One of ordinary skill will also appreciate that changes to  
the nucleotide sequences of the short repeat sequences of the  
donor DNA may coordinate with other mutation(s) in or near the  
15 binding region of the transposase enzyme to achieve the same  
increased binding effect, and the resulting 5- to 50-fold  
increase in transposition rate. Thus, while the applicants  
have exemplified one case of a mutation that improves binding  
of the exemplified transposase, it will be understood that  
20 other mutations in the transposase, or in the short repeat  
sequences, or in both, will also yield transposases that fall  
within the scope and spirit of the present invention. A  
suitable method for determining the relative avidity for Tn5 OE  
termini has been published by Jilk, R. A., et al., "The  
25 Organization of the Outside end of Transposon Tn5," J. Bact.  
178:1671-79 (1996).

The transposase of the present invention is also less  
likely than the wild type protein to assume an inactive  
multimeric form. In the preferred embodiment, that class (2)  
30 mutation from wild type can be achieved by modifying amino acid  
372 (leucine) of wild type Tn5 transposase to a proline (and,  
likewise by modifying the corresponding DNA to encode proline).  
This mutation, referred to as LP372, has previously been  
characterized as a mutation in the dimerization region of the  
35 transposase. Weinreich, et al., *supra*. It was noted by  
Weinreich et al. that this mutation at position 372 maps to a  
region shown previously to be critical for interaction with an  
inhibitor of Tn5 transposition. The inhibitor is a protein  
encoded by the same gene that encodes the transposase, but  
40 which is truncated at the N-terminal end of the protein,  
relative to the transposase. The approach of Weinreich et al.

5 for determining the extent to which multimers are formed is suitable for determining whether a mutation falls within the scope of this element.

10 It is thought that when wild type Tn5 transposase multimerizes, its activity *in trans* is reduced. Presumably, a mutation in the dimerization region reduces or prevents multimerization, thereby reducing inhibitory activity and leading to levels of transposition 5- to 50-fold higher than are seen with the wild type transposase. The LP372 mutation achieves about 10-fold higher transposition levels than wild type. Likewise, other mutations (including mutations at a one or more amino acid) that reduce the ability of the transposase to multimerize would also function in the same manner as the single mutation at position 372, and would also be suitable in a transposase of the present invention. It may also be possible to reduce the ability of a Tn5 transposase to multimerize without altering the wild type sequence in the so-called dimerization region, for example by adding into the system another protein or non-protein agent that blocks the dimerization site. Alternatively, the dimerization region could be removed entirely from the transposase protein.

25 As was noted above, the inhibitor protein, encoded in partially overlapping sequence with the transposase, can interfere with transposase activity. As such, it is desired that the amount of inhibitor protein be reduced over the amount observed in wild type *in vivo*. For the present assay, the transposase is used in purified form, and it may be possible to separate the transposase from the inhibitor (for example, according to differences in size) before use. However, it is also possible to genetically eliminate the possibility of having any contaminating inhibitor protein present by removing its start codon from the gene that encodes the transposase.

35 An AUG in the wild type Tn5 transposase gene that encodes methionine at transposase amino acid 56 is the first codon of the inhibitor protein. However, it has already been shown that replacement of the methionine at position 56 has no apparent effect upon the transposase activity, but at the same time

5 prevents translation of the inhibitor protein, thus resulting  
in a somewhat higher transposition rate. Weigand, T. W. and W.  
S. Reznikoff, "Characterization of Two Hypertransposing Tn5  
Mutants," J. Bact. 174:1229-1239 (1992), incorporated herein by  
reference. In particular, the present inventors have replaced  
10 the methionine with an alanine in the preferred embodiment (and  
have replaced the methionine-encoding AUG codon with an  
alanine-encoding GCC). A preferred transposase of the present  
invention therefore includes an amino acid other than  
methionine at amino acid position 56, although this change can  
15 be considered merely technically advantageous (since it ensures  
the absence of the inhibitor from the *in vitro* system) and not  
essential to the invention (since other means can be used to  
eliminate the inhibitor protein from the *in vitro* system).

The most preferred transposase amino acid sequence known  
20 to the inventors differs from the wild type at amino acid  
positions 54, 56, and 372. The mutations at positions 54 and  
372 separately contribute approximately a 10-fold increase to  
the rate of transposition reaction *in vivo*. When the mutations  
are combined using standard recombinant techniques into a  
25 single molecule containing both classes of mutations, reaction  
rates of at least about 100-fold higher than can be achieved  
using wild type transposase are observed when the products of  
the *in vitro* system are tested *in vivo*. The mutation at  
position 56 does not directly affect the transposase activity.

30 Other mutants from wild type that are contemplated to be  
likely to contribute to high transposase activity *in vitro*  
include, but are not limited to glutaminic acid-to-lysine at  
position 110, and glutamic acid to lysine at position 345.

It is, of course, understood that other changes apart from  
35 these noted positions can be made to the modified transposase  
(or to a construct encoding the modified transposase) without  
adversely affecting the transposase activity. For example, it  
is well understood that a construct encoding such a transposase  
could include changes in the third position of codons such that  
40 the encoded amino acid does not differ from that described  
herein. In addition, certain codon changes have little or no

5 functional effect upon the transposition activity of the  
encoded protein. Finally, other changes may be introduced  
which provide yet higher transposition activity in the encoded  
protein. It is also specifically envisioned that combinations  
10 of mutations can be combined to encode a modified transposase  
having even higher transposition activity than has been  
exemplified herein. All of these changes are within the scope  
of the present invention. It is noted, however, that a  
modified transposase containing the EK110 and EK345 mutations  
(both described by Weigand and Reznikoff, *supra*, had lower  
15 transposase activity than a transposase containing either  
mutation alone.

After the enzyme is prepared and purified, as described  
*supra*, it can be used in the *in vitro* transposition reaction  
described above to introduce any desired transposable element  
20 from a donor DNA into a target DNA. The donor DNA can be  
circular or can be linear. If the donor DNA is linear, it is  
preferred that the repeat sequences flanking the transposable  
element should not be at the termini of the linear fragment but  
should rather include some DNA upstream and downstream from the  
25 region flanked by the repeat sequences.

As was noted above, Tn5 transposition requires a pair of  
eighteen or nineteen base long termini. The wild type Tn5  
outside end (OE) sequence (5'-CTGACTCTTATACACAAGT-3') (SEQ ID  
NO: 7) has been described. It has been discovered that a  
30 transposase-catalyzed *in vitro* transposition frequency at least  
as high as that of wild type OE is achieved if the termini in a  
construct include bases ATA at positions 10, 11, and 12,  
respectively, as well as the nucleotides in common between wild  
type OE and IE (e.g., at positions 1-3, 5-9, 13, 14, 16, and  
35 optionally 19). The nucleotides at positions 4, 15, 17, and 18  
can correspond to the nucleotides found at those positions in  
either wild type OE or wild type IE. It is noted that the  
transposition frequency can be enhanced over that of wild type  
OE if the nucleotide at position 4 is a T. The importance of  
40 these particular bases to transposition frequency has not  
previously been identified.

5           It is noted that these changes are not intended to encompass every desirable modification to OE. As is described elsewhere herein, these attributes of acceptable termini modifications were identified by screening mutants having randomized differences between IE and OE termini. While the  
10       presence in the termini of certain nucleotides is shown herein to be advantageous, other desirable terminal sequences may yet be obtained by screening a larger array of degenerate mutants that include changes at positions other than those tested herein as well as mutants containing nucleotides not tested in  
15       the described screening. In addition, it is clear to one skilled in the art that if a different transposase is used, it may still be possible to select other variant termini, more compatible with that particular transposase.

20       Among the mutants shown to be desirable and within the scope of the invention are two hyperactive mutant OE sequences that were identified *in vivo*. Although presented here as single stranded sequences, in fact, the wild type and mutant OE sequences include complementary second strands. The first  
25       hyperactive mutant, 5'-CTGTCTCTTATACACATCT-3' (SEQ ID NO: 8), differs from the wild type OE sequence at positions 4, 17, and 18, counting from the 5' end, but retains ATA at positions 10-12. The second, 5'-CTGTCTCTTATACAGATCT-3' (SEQ ID NO: 9),  
30       differs from the wild type OE sequence at positions 4, 15, 17, and 18, but also retains ATA at positions 10-12. These two hyperactive mutant OE sequences differ from one another only at position 15, where either G or C is present. OE-like activity (or higher activity) is observed in a mutant sequence when it contains ATA at positions 10, 11 and 12. It may be possible to reduce the length of the OE sequence from 19 to 18 nucleotide  
35       pairs with little or no effect.

40       When one of the identified hyperactive mutant OE sequences flanks a substrate DNA, the *in vivo* transposition frequency of EK54/MA56 transposase is increased approximately 40-60 fold over the frequency that is observed when wild type OE termini flank the transposable DNA. The EK54/MA56 transposase is already known to have an *in vivo* transposition frequency



5 approximately an 8-10 fold higher than wild type transposase, using wild type OE termini. Tn5 transposase having the EK54/MA56 mutation is known to bind with greater avidity to OE and with lesser avidity to the Tn5 inside ends (IE) than wild type transposase.

10 A suitable mutant terminus in a construct for use in the assays of the present invention is characterized biologically as yielding more papillae per colony in a comparable time, say 68 hours, than is observed in colonies harboring wild type OE in a comparable plasmid. Wild type OE can yield about 100  
15 papillae per colony when measured 68 hours after plating in a papillation assay using EK54/MA56 transposase, as is described elsewhere herein. A preferred mutant would yield between about 200 and 3000 papillae per colony, and a more preferred mutant between about 1000 and 3000 papillae per colony, when measured  
20 in the same assay and time frame. A most preferred mutant would yield between about 2000 and 3000 papillae per colony when assayed under the same conditions. Papillation levels may be even greater than 3000 per colony, although it is difficult to quantitate at such levels.

25 Transposition frequency is also substantially enhanced in the *in vitro* transposition assay of the present invention when substrate DNA is flanked by a preferred mutant OE sequence and a most preferred mutant transposase (comprising EK54/MA56/LP372 mutations) is used. Under those conditions, essentially all of  
30 the substrate DNA is converted into transposition products.

The rate of *in vitro* transposition observed using the hyperactive termini is sufficiently high that, in the experience of the inventors, there is no need to select for  
35 transposition events. All colonies selected at random after transformation for further study have shown evidence of transposition events.

This advance can represent a significant savings in time and laboratory effort. For example, it is particularly advantageous to be able to improve *in vitro* transposition  
40 frequency by modifying DNA rather than by modifying the transposase because as transposase activity increases in host

5 cells, there is an increased likelihood that cells containing the transposase are killed during growth as a result of aberrant DNA transpositions. In contrast, DNA of interest containing the modified OE termini can be grown in sources completely separate from the transposase, thus not putting the  
10 host cells at risk.

Without intending to limit the scope of this aspect of this invention, it is apparent that the tested hyperactive termini do not bind with greater avidity to the transposase than do wild type OE termini. Thus, the higher transposition  
15 frequency brought about by the hyperactive termini is not due to enhanced binding to transposase.

The transposable element between the termini can include any desired nucleotide sequence. The length of the transposable element between the termini should be at least  
20 about 50 base pairs, although smaller inserts may work. No upper limit to the insert size is known. However, it is known that a donor DNA portion of about 300 nucleotides in length can function well. By way of non-limiting examples, the transposable element can include a coding region that encodes a  
25 detectable or selectable protein, with or without associated regulatory elements such as promoter, terminator, or the like.

If the element includes such a detectable or selectable coding region without a promoter, it will be possible to identify and map promoters in the target DNA that are uncovered  
30 by transposition of the coding region into a position downstream thereof, followed by analysis of the nucleic acid sequences upstream from the transposition site.

Likewise, the element can include a primer binding site that can be transposed into the target DNA, to facilitate  
35 sequencing methods or other methods that rely upon the use of primers distributed throughout the target genetic material. Similarly, the method can be used to introduce a desired restriction enzyme site or polylinker, or a site suitable for another type of recombination, such as a cre-lox, into the  
40 target.

The invention can be better understood upon consideration

5 of the following examples which are intended to be exemplary and not limiting on the invention.

#### EXAMPLES

10 To obtain the transposase modified at position 54, the first third of the coding region from an existing DNA clone that encodes the Tn5 transposase but not the inhibitor protein (MA56) was mutagenized according to known methods and DNA fragments containing the mutagenized portion were cloned to produce a library of plasmid clones containing a full length transposase gene. The clones making up the library were  
15 transformed into *E. coli* K-12 strain MDW320 bacteria which were plated and grown into colonies. Transposable elements provided in the bacteria on a separate plasmid contained a defective lacZ gene. The separate plasmid, pOXgen386, was described by Weinreich, M. et al., "A functional analysis of the Tn5  
20 Transposase: Identification of Domains Required for DNA Binding and Dimerization," J. Mol. Biol. 241:166-177 (1993), incorporated herein by reference. Colonies having elevated transposase activity were selected by screening for blue (LacZ) spots in white colonies grown in the presence of X-gal. This  
25 papillation assay was described by Weinreich, et al. (1993), supra. The 5'-most third of Tn5 transposase genes from such colonies were sequenced to determine whether a mutation was responsible for the increase in transposase activity. It was determined that a mutation at position 54 to lysine (K) correlated well with the increase in transposase activity.  
30 Plasmid pRZ5412-EK54 contains lysine at position 54 as well as the described alanine at position 56.

The fragment containing the LP372 mutation was isolated from pRZ4870 (Weinreich et al (1994)) using restriction enzymes  
35 NheI and BglIII, and were ligated into NheI-BglIII cut pRZ5412-EK54 to form a recombinant gene having the mutations at positions 54, 56 and 372, as described herein and shown in SEQ ID NO:1. The gene was tested and shown to have at least about a one hundred fold increase in activity relative to wild type  
40 Tn5 transposase. Each of the mutants at positions 54 and 372

5 alone had about a 10-fold increase in transposase activity.

The modified transposase protein encoded by the triple-mutant recombinant gene was transferred into commercial T7 expression vector pET-21D (commercially available from Novagen, Madison, WI) by inserting a BspHI/SalI fragment into NhoI/XhoI  
10 fragment of the pET-21D vector. This cloning puts the modified transposase gene under the control of the T7 promoter, rather than the natural promoter of the transposase gene. The gene product was overproduced in BL21(DE3)pLyss bacterial host cells, which do not contain the binding site for the enzyme, by  
15 specific induction in a fermentation process after cell growth is complete. (See, Studier, F. W., et al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Methods Enzymol. 185:60-89 (1990)). The transposase was partially purified using the method of de la Cruz, modified by inducing  
20 overproduction at 33 or 37°C. After purification, the enzyme preparation was stored at -70°C in a storage buffer (10% glycerol, 0.7M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Triton-X100 and 10 mM CHAPS) until use. This storage buffer is to be considered exemplary and not optimized.

25 A single plasmid (pRZTL1, Fig. 1) was constructed to serve as both donor and target DNA in this Example. The complete sequence of the pRZTL1 plasmid DNA is shown and described in SEQ ID NO:3. Plasmid pRZTL1 contains two Tn5 19 base pair OE termini in inverted orientation to each other. Immediately  
30 adjacent to one OE sequence is a gene that would encode tetracycline resistance, but for the lack of an upstream promoter. However, the gene is expressed if the tetracycline resistance gene is placed downstream of a transcribed region (e.g., under the control of the promoter that promotes  
35 transcription of the chloramphenicol resistance gene also present on pRZTL1). Thus, the test plasmid pRZTL1 can be assayed *in vivo* after the *in vitro* reaction to confirm that transposition has occurred. The plasmid pRZTL1 also includes  
40 an origin of replication in the transposable element, which ensures that all transposition products are plasmids that can replicate after introduction in host cells.

5           The following components were used in typical 20 $\mu$ l *in vitro* transposition reactions:

Modified transposase: 2  $\mu$ l (approximately 0.1  $\mu$ g enzyme/ $\mu$ l) in storage buffer (10% glycerol, 0.7M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Triton-X100 and 10 mM CHAPS)

10           Donor/Target DNA: 18  $\mu$ l (approximately 1-2  $\mu$ g) in reaction buffer (at final reaction concentrations of 0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 10 mM Mg<sup>2+</sup>-acetate, 50  $\mu$ g/ml BSA, 0.5 mM  $\beta$ -mercaptoethanol, 2 mM spermidine, 100  $\mu$ g/ml tRNA).

15           At 20°C, the transposase was combined with pRZTL1 DNA for about 60 minutes. Then, the reaction volume was increased by adding two volumes of reaction buffer and the temperature was raised to 37°C for 2-3 hours whereupon cleavage and strand transfer occurred.

20           Efficient *in vitro* transposition was shown to have occurred by *in vivo* and by *in vitro* methods. *In vivo*, many tetracycline-resistant colonies were observed after transferring the nucleic acid product of the reaction into DH5 $\alpha$  bacterial cells. As noted, tetracycline resistance can only  
25           arise in this system if the transposable element is transposed downstream from an active promoter elsewhere on the plasmid. A typical transposition frequency was 0.1% of cells that received plasmid DNA, as determined by counting chloramphenicol  
30           resistant colonies. However, this number underestimates the total transposition event frequency because the detection system limits the target to 1/16 of the total.

35           Moreover, *in vitro* electrophoretic (1% agarose) and DNA sequencing analyses of DNA isolated from purified colonies revealed products of true transposition events, including both intramolecular and intermolecular events. Results of typical reactions using circular plasmid pRZTL1 substrates are shown in  
Lanes 4 & 5. Lane 6 of Fig. 2 shows the results obtained using linear plasmid pRZTL1 substrates.

5 The bands were revealed on 1% agarose gels by staining with SYBR Green (FMC BioProducts) and were scanned on a Fluorimager SI (Molecular Dynamics). In Figure 2, lane 1 shows relaxed circle, linear, and closed circle versions of pRZTL1. Lanes 2 and 3 show intramolecular and intermolecular  
10 transposition products after *in vitro* transposition of pRZTL1, respectively. The products were purified from electroporated DH5 $\alpha$  cells and were proven by size and sequence analysis to be genuine transposition products. Lanes 4 and 5 represent products of two independent *in vitro* reactions using a mixture  
15 of closed and relaxed circular test plasmid substrates. In lane 6, linear pRZTL1 (XhoI-cut) was the reaction substrate. Lane 7 includes a BstEII digest of lambda DNA as a molecular weight standard.

20 Fig. 3 reproduces lanes 4, 5, and 6 of Fig. 2 and shows an analysis of various products, based upon secondary restriction digest experiments and re-electroporation and DNA sequencing. The released donor DNA corresponds to the fragment of pRZTL1 that contains the kanamycin resistance gene between the two OE sequences, or, in the case of the linear substrate, the OE-XhoI  
25 fragment. Intermolecular transposition products can be seen only as relaxed DNA circles. Intramolecular transposition products are seen as a ladder, which results from conversion of the initial superhelicity of the substrate into DNA knots. The reaction is efficient enough to achieve double transposition  
30 events that are a combination of inter- and intramolecular events.

A preliminary investigation was made into the nature of the termini involved in a transposition reaction. Wild type Tn5 OE and IE sequences were compared and an effort was  
35 undertaken to randomize the nucleotides at each of the seven positions of difference. A population of oligonucleotides degenerate at each position of difference was created. Thus, individual oligonucleotides in the population randomly included either the nucleotide of the wild type OE or the wild type IE  
40 sequence. In this scheme, 2<sup>7</sup> (128) distinct oligonucleotides were synthesized using conventional tools. These

oligonucleotides having sequence characteristics of both OE and IE are referred to herein as OE/IE-like sequences. To avoid nomenclature issues that arise because the oligonucleotides are intermediate between OE and IE wild type sequences, the applicants herein note that selected oligonucleotide sequences are compared to the wild type OE rather than to wild type IE, unless specifically noted. It will be appreciated by one skilled in the art that if IE is selected as the reference point, the differences are identical but are identified differently.

The following depicts the positions (x) that were varied in this mutant production scheme. WT OE is shown also at SEQ ID NO: 7, WT IE at SEQ ID NO: 10.

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5'-CTGACTCTTATACACAAGT-3' (WT OE)
      x      xxx  x  xx      (positions of difference)
5'-CTGTCTCTTGATCAGATCT-3' (WT IE)

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In addition to the degenerate OE/IE-like sequences, the 37- base long synthetic oligonucleotides also included terminal *Sph*I and *Kpn*I restriction enzyme recognition and cleavage sites for convenient cloning of the degenerate oligonucleotides into plasmid vectors. Thus, a library of randomized termini was created from population of 2<sup>7</sup> (128) types of degenerate oligonucleotides.

Fig. 4 shows pRZ1496, the complete sequence of which is presented as SEQ ID NO:11. The following features are noted in the sequence:

<u>Feature</u>	<u>Position</u>
WT OE	94-112
LacZ coding	135-3137
LacY coding	3199-4486
LacA coding	4553-6295
tet <sup>r</sup> coding	6669-9442
transposase coding	10683-12111 (Comp. Strand)
Cassette IE	12184-12225
colE1 sequence	127732-19182

The IE cassette shown in Fig. 4 was excised using *Sph*I and *Kpn*I and was replaced, using standard cleavage and ligation methods, by the synthetic termini cassettes comprising OE/IE-

5 like portions. Between the fixed wild type OE sequence and the  
OE/IE-like cloned sequence, plasmid pRZ1496 comprises a gene  
whose activity can be detected, namely LacZ<sup>+</sup>YA, as well as a  
selectable marker gene, tet<sup>r</sup>. The LacZ gene is defective in  
that it lacks suitable transcription and translation initiation  
10 signals. The LacZ gene is transcribed and translated only when  
it is transposed into a position downstream from such signals.

The resulting clones were transformed using  
electroporation into dam<sup>-</sup>, LacZ<sup>-</sup> bacterial cells, in this case  
JCM101/pOXgen cells which were grown at 37°C in LB medium under  
15 standard conditions. A dam<sup>-</sup> strain is preferred because dam  
methylation can inhibit IE utilization and wild type IE  
sequences include two dam methylation sites. A dam<sup>-</sup> strain  
eliminates dam methylation as a consideration in assessing  
transposition activity. The Tet<sup>r</sup> cells selected were LacZ<sup>-</sup>;  
20 transposition-activated Lac expression was readily detectable  
against a negative background. pOXgen is a non-essential F  
factor derivative that need not be provided in the host cells.

In some experiments, the EK54/MA56 transposase was encoded  
directly by the transformed pRZ1496 plasmid. In other  
25 experiments, the pRZ1496 plasmid was modified by deleting a  
unique HindIII/EagI fragment (nucleotides 9112-12083) from the  
plasmid (see Fig. 4) to prevent transposase production. In the  
latter experiments, the host cells were co-transformed with the  
HindIII/EagI-deleted plasmid, termed pRZ5451 (Fig. 4), and with  
30 an EK54/MA56 transposase-encoding chloramphenicol-resistant  
plasmid. In some experiments, a comparable plasmid encoding a  
wild type Tn5 transposase was used for comparison.

Transposition frequency was assessed by a papillation  
assay that measured the number of blue spots (Lac producing  
35 cells or "papillae") in an otherwise white colony. Transformed  
cells were plated (approx. 50 colonies per plate) on Glucose  
minimal Miller medium (Miller, J., Experiments in Molecular  
Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY  
(1972)) containing 0.3% casamino acids, 5-bromo-4-chloro-3-  
40 indolyl- $\beta$ -D-galactoside (40  $\mu$ g/ml) and phenyl- $\beta$ -D-galactoside  
(0.05%). The medium contained tetracycline (15  $\mu$ g/ml) and,



5 where needed, chloramphenicol (20  $\mu$ g/ml). Colonies that survived the selection were evaluated for transposition frequency *in vivo*. Although colonies exhibiting superior papillation were readily apparent to the naked eye, the number of blue spots per colony were determined over a period of  
10 several days (approximately 90 hours post-plating).

To show that the high-papillation phenotype was conferred by the end mutations in the plasmids, colonies were re-streaked if they appeared to have papillation levels higher than was observed when wild type IE was included on the plasmid.  
15 Colonies picked from the streaked culture plates were themselves picked and cultured. DNA was obtained and purified from the cultured cells, using standard protocols, and was transformed again into "clean" JCM101/pOXgen cells. Papillation levels were again compared with wild type IE-  
20 containing plasmids in the above-noted assays, and consistent results were observed.

To obtain DNA for sequencing of the inserted oligonucleotide, cultures were grown from white portions of 117 hyperpapillating colonies, and DNA was prepared from each  
25 colony using standard DNA miniprep methods. The DNA sequence of the OE/IE-like portion of 117 clones was determined (42 from transformations using pRZ1496 as the cloning vehicle; 75 from transformations using pRZ5451 as the cloning vehicle). Only 29 unique mutants were observed. Many mutants were isolated  
30 multiple times. All mutants that showed the highest papillation frequencies contain OE-derived bases at positions 10, 11, and 12. When the OE-like bases at these positions were maintained, it was impossible to measure the effect on transposition of other changes, since the papillation level was  
35 already extremely high.

One thousand five hundred seventy five colonies were screened as described above. The likelihood that all 128 possible mutant sequences were screened was greater than 95%. Thus, it is unlikely that other termini that contribute to a  
40 greater transformation frequency will be obtained using the tested transposase.

TABLE I.  
trans papillation level of hybrid end sequences with EK54 Tnp

mutant	position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1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TABLE II.  
cis papillation level of hybrid end sequences with EK54 Tnp

mutant	position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	papillation level <sup>a</sup>	# of times isolated <sup>b</sup>
IE		c	t	g	T	c	t	c	t	t	G	A	T	A	T	c	a	T	C	t	L	0
OE					A						A	T	A	A				A	A		H	2
1											A	T	A	A							H	2
2											A	T	A	A				A			H	0
3											A	T	A	A				A			H	1
4											A	T	A	A				A			H	1
5											A	T	A	A					G		H	1
6											A	T	A	A				A	G		H	2
7											A	T	A	A				A	G		H	3
8											A	T	A	A				A	G		H	1
9											A	T	A	A							H	1
10					A						A	T	A	A				A			H	0
11				A	A						A	T	A	A					G		H	2
12				A	A						A	T	A	A				A			MH	3
13				A	A						A	T	A	A				A			MH	1
14				A	A						A	T	A	A					G		H	0
15				A	A						A	T	A	A				A	G		H	2
16											A	T	A	A							M	1
17											A	T	A	A				A	G		M	1
18											A	T	A	A							M	2
19				A							A	T	A	A				A			M	2
20				A							A	T	A	A				A	G		M	1
21				A							A	T	A	A				A			M	4
22											A	T	A	A					G		M	1
23											A	T	A	A				A	G		M	1
24											A	T	A	A				A			M	1
25											A	T	A	A				A	G		M	1
26																		A	G		M	1
27												T						A	G		M	2

All hybrid end sequences isolated on PRZ1496 that papillate more frequently than wt IE, when the EK54 Tnp is expressed from the same plasmid, are listed. <sup>a</sup>cis papillation levels of wt IE, wt OE and hybrid end sequences are classified as follows: L-low, M-medium, MH-medium high, and H-high. <sup>b</sup>Although mutants 2, 10 and 14 were not found in this experiment, they were found in trans papillation screening (Table I).

5           Tables I and II report the qualitative papillation level  
of mutant constructs carrying the indicated hybrid end  
sequences or the wild type OE or IE end sequences. In the  
tables, the sequence at each position of the terminus  
corresponds to wild type IE unless otherwise noted. The  
10       applicants intend that, while the sequences are presented in  
shorthand notation, one of ordinary skill can readily determine  
the complete 19 base pair sequence of every presented mutant,  
and this specification is to be read to include all such  
complete sequences. Table I includes data from trials where  
15       the EK54 transposase was provided *in trans*; Table II, from  
those trials where the EK54 transposase was provided *in cis*.  
Although a transposase provided *in cis* is more active in  
absolute terms than a transposase provided *in trans*, the *cis* or  
*trans* source of the transposase does not alter the relative *in*  
20       vivo transposition frequencies of the tested termini.

Tables I and II show that every mutant that retains ATA at  
positions 10, 11, and 12, respectively, had an activity  
comparable to, or higher than, wild type OE, regardless of  
whether the wild type OE activity was medium (Table I, *trans*)  
25       or high (Table II, *cis*). Moreover, whenever that three-base  
sequence in a mutant was not ATA, the mutant exhibited lower  
papillation activity than wild type OE. It was also noted that  
papillation is at least comparable to, and tends to be  
significantly higher than, wild type OE when position 4 is a T.

30       Quantitative analysis of papillation levels was difficult,  
beyond the comparative levels shown (very low, low, medium,  
medium high, and high). However, one skilled in the art can  
readily note the papillation level of OE and can recognize  
those colonies having comparable or higher levels. It is  
35       helpful to observe the papillae with magnification.

The number of observed papillae increased over time, as is  
shown in Figs 5 - 7 which roughly quantitate the papillation  
observed in cells transformed separately with 9 clones  
containing either distinct synthetic termini cassettes or wild  
40       type OE or IE termini. In these 3 figures, each mutant is  
identified by its differences from the wild type IE sequence.

5 Note that, among the tested mutants, only mutant 10A/11T/12A  
had a higher transposition papillation level than wild type OE.  
That mutant, which would be called mutant 4/15/17/18 when OE is  
the reference sequence) was the only mutant of those shown in  
10 Figs. 5-7 that retained the nucleotides ATA at positions 10,  
11, and 12. Figs. 5 (y-axis: 0 - 1500 papillae) and 6 (y-axis:  
0 - 250 papillae) show papillation using various mutants plus  
IE and OE controls and the EK54/MA56 enzyme. Fig. 7 (y-axis: 0  
- 250 papillae), shows papillation when the same mutant  
sequences were tested against the wild type (more properly,  
15 MA56) transposase. The 10A/11T/12A mutant (SEQ ID NO: 9)  
yielded significantly more papillae (approximately 3000) in a  
shorter time (68 hours) with ED54/MA56 transposase than was  
observed even after 90 hours with the WT OE (approximately  
1500). A single OE-like nucleotide at position 15 on an IE-  
20 like background also increased papillation frequency.

In vivo transposition frequency was also quantitated in a  
tetracycline-resistance assay using two sequences having high  
levels of hyperpapillation. These sequences were 5'-  
CTGTCTCTTATACACATCT-3' (SEQ ID NO: 8), which differs from the  
25 wild type OE sequence at positions 4, 17, and 18, counting from  
the 5' end, and 5'-CTGTCTCTTATACAGATCT-3' (SEQ ID NO: 9), which  
differs from the wild type OE at positions 4, 15, 17, and 18.  
These sequences are considered the preferred mutant termini in  
an assay using a transposase that contains EK54/MA56 or a  
30 transposase that contains MA56. Each sequence was separately  
engineered into pRZTL1 in place of the plasmid's two wild type  
OE sequences. A PCR-amplified fragment containing the desired  
ends flanking the kanamycin resistance gene was readily cloned  
into the large HindIII fragment of pRZTL1. The resulting  
35 plasmids are identical to pRZTL1 except at the indicated  
termini. For comparison, pRZTL1 and a derivative of pRZTL1  
containing two wild type IE sequences were also tested. In the  
assay, JCM101/pOXgen cells were co-transformed with a test  
plasmid (pRZTL1 or derivative) and a high copy number amp<sup>r</sup>  
40 plasmid that encoded either the EK54/MA56 transposase or wild  
type (MA56) transposase. The host cells become tetracycline

5 resistant only when a transposition event brings the Tet<sup>r</sup> gene  
into downstream proximity with a suitable transcriptional  
promoter elsewhere on a plasmid or on the chromosome. The  
total number of cells that received the test plasmids was  
determined by counting chloramphenicol resistant, ampicillin  
10 resistant colonies. Transposition frequency was calculated by  
taking the ratio of tet<sup>r</sup>/cam<sup>r</sup>amp<sup>r</sup> colonies. Approximately 40 to  
60 fold increase over wild type OE in *in vivo* transposition was  
observed when using either of the mutant termini and EK54/MA56  
transposase. Of the two preferred mutant termini, the one  
15 containing mutations at three positions relative to the wild  
type OE sequence yielded a higher increase.

As is shown in Fig. 8, which plots the tested plasmid  
against the transposition frequency ( $\times 10^{-8}$ ), little  
transposition was seen when the test plasmid included two IE  
20 termini. Somewhat higher transposition was observed when the  
test plasmid included two OE termini, particularly when the  
EK54/MA56 transposase was employed. In striking contrast, the  
combination of the EK54/MA56 transposase with either of the  
preferred selected ends (containing OE-like bases only at  
25 positions 10, 11, and 12, or positions 10, 11, 12, and 15)  
yielded a great increase in *in vivo* transposition over wild  
type OE termini.

The preferred hyperactive mutant terminus having the most  
preferred synthetic terminus sequence 5'-CTGTCTCTTATACACATCT-3'  
30 (SEQ ID NO: 8) was provided in place of both WT OE termini in  
pRZTL1 (Fig. 4) and was tested in the *in vitro* transposition  
assay of the present invention using the triple mutant  
transposase described herein. This mutant terminus was chosen  
for further *in vitro* analysis because its transposition  
35 frequency was higher than for the second preferred synthetic  
terminus and because it has no dam methylation sites, so dam  
methylation no longer affects transposition frequency. In  
contrast the 4/15/17/18 mutant does have a dam methylation  
site.

40 In a preliminary experiment, CHAPS was eliminated from the  
reaction, but the pre-incubation step was used. The reaction

5 was pre-incubated for 1 hour at 20°C, then diluted two times,  
and then incubated for 3 hours at 37°C. About 0.5 µg of DNA  
and 0.4µg of transposase was used. The transposition products  
10 were observed on a gel. With the mutant termini, very little  
of the initial DNA was observed. Numerous bands representing  
primary and secondary transposition reaction products were  
observed. The reaction mixtures were transformed into DH5α  
cells and were plated on chloramphenicol-, tetracycline-, or  
kanamycin-containing plates.

15 Six hundred forty chloramphenicol-resistant colonies were  
observed. Although these could represent unreacted plasmid,  
all such colonies tested (n=12) were sensitive to kanamycin,  
which indicates a loss of donor backbone DNA. All twelve  
colonies also included plasmids of varied size; 9 of the 12  
20 were characterized as deletion-inversions, the remaining 3 were  
simple deletions. Seventy nine tetracycline-resistant  
colonies were observed, which indicated an activation of the  
tet<sup>r</sup> gene by transposition.

Eleven kanamycin resistant colonies were observed. This  
indicated a low percentage of remaining plasmids carrying the  
25 donor backbone DNA.

In a second, similar test, about 1 µg of plasmid DNA and  
0.2 µg transposase were used. In this test, the reaction was  
incubated without CHAPS at 37°C for 3 hours without pre-  
incubation or dilution. Some initial DNA was observed in the  
30 gel after the 3 hour reaction. After overnight incubation,  
only transposition products were observed.

The 3 hour reaction products were transformed into DH5α  
cells and plated as described. About 50% of the  
chloramphenicol resistant colonies were sensitive to kanamycin  
35 and were presumably transposition products.

The invention is not intended to be limited to the  
foregoing examples, but to encompass all such modifications and  
variations as come within the scope of the appended claims.  
It is envisioned that, in addition to the uses specifically  
40 noted herein, other applications will be apparent to the  
skilled molecular biologist. In particular, methods for

5 introducing desired mutations into prokaryotic or eukaryotic  
DNA are very desirable. For example, at present it is  
difficult to knock out a functional eukaryotic gene by  
homologous recombination with an inactive version of the gene  
that resides on a plasmid. The difficulty arises from the need  
10 to flank the gene on the plasmid with extensive upstream and  
downstream sequences. Using this system, however, an  
inactivating transposable element containing a selectable  
marker gene (e.g., neo) can be introduced in vitro into a  
plasmid that contains the gene that one desires to inactivate.  
15 After transposition, the products can be introduced into  
suitable host cells. Using standard selection means, one can  
recover only cell colonies that contain a plasmid having the  
transposable element. Such plasmids can be screened, for  
example by restriction analysis, to recover those that contain  
20 a disrupted gene. Such clones can then be introduced directly  
into eukaryotic cells for homologous recombination and  
selection using the same marker gene.

Also, one can use the system to readily insert a PCR-  
amplified DNA fragment into a vector, thus avoiding traditional  
25 cloning steps entirely. This can be accomplished by (1)  
providing suitable a pair of PCR primers containing OE termini  
adjacent to the sequence-specific parts of the primers, (2)  
performing standard PCR amplification of a desired nucleic acid  
fragment, (3) performing the in vitro transposition reaction of  
30 the present invention using the double-stranded products of PCR  
amplification as the donor DNA.



5

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Reznikoff, William S  
Gorysin, Igor Y  
Zhou, Hong

10

(ii) TITLE OF INVENTION: System for In Vitro Transposition

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: Quarles & Brady  
(B) STREET: 1 South Pinckney Street  
(C) CITY: Madison  
(D) STATE: WI  
(E) COUNTRY: USA  
(F) ZIP: 53703

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

30

(vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Berson, Bennett J  
(B) REGISTRATION NUMBER: 37094  
(C) REFERENCE/DOCKET NUMBER: 960296.94142

(ix) TELECOMMUNICATION INFORMATION:

35

(A) TELEPHONE: 608/251-5000  
(B) TELEFAX: 608-251-9166

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

40

(A) LENGTH: 1534 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Gene encoding modified Tn5  
transposase enzyme"

45

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 93..1523

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGACTCTTA TACACAAGTA GCGTCCTGAA CGGAACCTTT CCCGTTTTCC AGGATCTGAT 60

50

CTTCCATGTG ACCTCCTAAC ATGGTAACGT TC ATG ATA ACT TCT GCT CTT CAT 113  
Met Ile Thr Ser Ala Leu His

1

5

5	CGT GCG GCC GAC TGG GCT AAA TCT GTG TTC TCT TCG GCG GCG CTG GGT Arg Ala Ala Asp Trp Ala Lys Ser Val Phe Ser Ser Ala Ala Leu Gly 10 15 20	161
10	GAT CCT CGC CGT ACT GCC CGC TTG GTT AAC GTC GCC GCC CAA TTG GCA Asp Pro Arg Arg Thr Ala Arg Leu Val Asn Val Ala Ala Gln Leu Ala 25 30 35	209
	AAA TAT TCT GGT AAA TCA ATA ACC ATC TCA TCA GAG GGT AGT AAA GCC Lys Tyr Ser Gly Lys Ser Ile Thr Ile Ser Ser Glu Gly Ser Lys Ala 40 45 50 55	257
15	GCC CAG GAA GGC GCT TAC CGA TTT ATC CGC AAT CCC AAC GTT TCT GCC Ala Gln Glu Gly Ala Tyr Arg Phe Ile Arg Asn Pro Asn Val Ser Ala 60 65 70	305
	GAG GCG ATC AGA AAG GCT GGC GCC ATG CAA ACA GTC AAG TTG GCT CAG Glu Ala Ile Arg Lys Ala Gly Ala Met Gln Thr Val Lys Leu Ala Gln 75 80 85	353
20	GAG TTT CCC GAA CTG CTG GCC ATT GAG GAC ACC ACC TCT TTG AGT TAT Glu Phe Pro Glu Leu Leu Ala Ile Glu Asp Thr Thr Ser Leu Ser Tyr 90 95 100	401
25	CGC CAC CAG GTC GCC GAA GAG CTT GGC AAG CTG GGC TCT ATT CAG GAT Arg His Gln Val Ala Glu Glu Leu Gly Lys Leu Gly Ser Ile Gln Asp 105 110 115	449
	AAA TCC CGC GGA TGG TGG GTT CAC TCC GTT CTC TTG CTC GAG GCC ACC Lys Ser Arg Gly Trp Trp Val His Ser Val Leu Leu Leu Glu Ala Thr 120 125 130 135	497
30	ACA TTC CGC ACC GTA GGA TTA CTG CAT CAG GAG TGG TGG ATG CGC CCG Thr Phe Arg Thr Val Gly Leu Leu His Gln Glu Trp Trp Met Arg Pro 140 145 150	545
	GAT GAC CCT GCC GAT GCG GAT GAA AAG GAG AGT GGC AAA TGG CTG GCA Asp Asp Pro Ala Asp Ala Asp Glu Lys Glu Ser Gly Lys Trp Leu Ala 155 160 165	593
35	GCG GCC GCA ACT AGC CGG TTA CGC ATG GGC AGC ATG ATG AGC AAC GTG Ala Ala Ala Thr Ser Arg Leu Arg Met Gly Ser Met Met Ser Asn Val 170 175 180	641
40	ATT GCG GTC TGT GAC CGC GAA GCC GAT ATT CAT GCT TAT CTG CAG GAC Ile Ala Val Cys Asp Arg Glu Ala Asp Ile His Ala Tyr Leu Gln Asp 185 190 195	689
	AGG CTG GCG CAT AAC GAG CGC TTC GTG GTG CGC TCC AAG CAC CCA CGC Arg Leu Ala His Asn Glu Arg Phe Val Val Arg Ser Lys His Pro Arg 200 205 210 215	737
45	AAG GAC GTA GAG TCT GGG TTG TAT CTG ATC GAC CAT CTG AAG AAC CAA Lys Asp Val Glu Ser Gly Leu Tyr Leu Ile Asp His Leu Lys Asn Gln 220 225 230	785
	CCG GAG TTG GGT GGC TAT CAG ATC AGC ATT CCG CAA AAG GGC GTG GTG Pro Glu Leu Gly Gly Tyr Gln Ile Ser Ile Pro Gln Lys Gly Val Val 235 240 245	833
50	GAT AAA CGC GGT AAA CGT AAA AAT CGA CCA GCC CGC AAG GCG AGC TTG Asp Lys Arg Gly Lys Arg Lys Asn Arg Pro Ala Arg Lys Ala Ser Leu 250 255 260	881
55	AGC CTG CGC AGT GGG CGC ATC ACG CTA AAA CAG GGG AAT ATC ACG CTC Ser Leu Arg Ser Gly Arg Ile Thr Leu Lys Gln Gly Asn Ile Thr Leu 265 270 275	929

5	AAC GCG GTG CTG GCC GAG GAG ATT AAC CCG CCC AAG GGT GAG ACC CCG Asn Ala Val Leu Ala Glu Glu Ile Asn Pro Pro Lys Gly Glu Thr Pro 280 285 290 295	977
10	TTG AAA TGG TTG TTG CTG ACC GGC GAA CCG GTC GAG TCG CTA GCC CAA Leu Lys Trp Leu Leu Leu Thr Gly Glu Pro Val Glu Ser Leu Ala Gln 300 305 310	1025
15	GCC TTG CGC GTC ATC GAC ATT TAT ACC CAT CGC TGG CGG ATC GAG GAG Ala Leu Arg Val Ile Asp Ile Tyr Thr His Arg Trp Arg Ile Glu Glu 315 320 325	1073
20	TTC CAT AAG GCA TGG AAA ACC GGA GCA GGA GCC GAG AGG CAA CGC ATG Phe His Lys Ala Trp Lys Thr Gly Ala Gly Ala Glu Arg Gln Arg Met 330 335 340	1121
25	GAG GAG CCG GAT AAT CTG GAG CGG ATG GTC TCG ATC CTC TCG TTT GTT Glu Glu Pro Asp Asn Leu Glu Arg Met Val Ser Ile Leu Ser Phe Val 345 350 355	1169
30	GCG GTC AGG CTG TTA CAG CTC AGA GAA AGC TTC ACG CCG CCG CAA GCA Ala Val Arg Leu Leu Gln Leu Arg Glu Ser Phe Thr Pro Pro Gln Ala 360 365 370 375	1217
35	CTC AGG GCG CAA GGG CTG CTA AAG GAA GCG GAA CAC GTA GAA AGC CAG Leu Arg Ala Gln Gly Leu Leu Lys Glu Ala Glu His Val Glu Ser Gln 380 385 390	1265
40	TCC GCA GAA ACG GTG CTG ACC CCG GAT GAA TGT CAG CTA CTG GGC TAT Ser Ala Glu Thr Val Leu Thr Pro Asp Glu Cys Gln Leu Leu Gly Tyr 395 400 405	1313
45	CTG GAC AAG GGA AAA CGC AAG CGC AAA GAG AAA GCA GGT AGC TTG CAG Leu Asp Lys Gly Lys Arg Lys Arg Lys Glu Lys Ala Gly Ser Leu Gln 410 415 420	1361
50	TGG GCT TAC ATG GCG ATA GCT AGA CTG GGC GGT TTT ATG GAC AGC AAG Trp Ala Tyr Met Ala Ile Ala Arg Leu Gly Gly Phe Met Asp Ser Lys 425 430 435	1409
55	CGA ACC GGA ATT GCC AGC TGG GGC GCC CTC TGG GAA GGT TGG GAA GCC Arg Thr Gly Ile Ala Ser Trp Gly Ala Leu Trp Glu Gly Trp Glu Ala 440 445 450 455	1457
60	CTG CAA AGT AAA CTG GAT GGC TTT CTT GCC GCC AAG GAT CTG ATG GCG Leu Gln Ser Lys Leu Asp Gly Phe Leu Ala Ala Lys Asp Leu Met Ala 460 465 470	1505
65	CAG GGG ATC AAG ATC TGA TCAAGAGACA G Gln Gly Ile Lys Ile *	1534

## (2) INFORMATION FOR SEQ ID NO:2:

## 45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ile Thr Ser Ala Leu His Arg Ala Ala Asp Trp Ala Lys Ser Val  
1 5 10 15

5 Phe Ser Ser Ala Ala Leu Gly Asp Pro Arg Arg Thr Ala Arg Leu Val  
                     20                    25                    30  
 Asn Val Ala Ala Gln Leu Ala Lys Tyr Ser Gly Lys Ser Ile Thr Ile  
                     35                    40                    45  
 10 Ser Ser Glu Gly Ser Lys Ala Ala Gln Glu Gly Ala Tyr Arg Phe Ile  
                     50                    55                    60  
 Arg Asn Pro Asn Val Ser Ala Glu Ala Ile Arg Lys Ala Gly Ala Met  
                     65                    70                    75                    80  
 Gln Thr Val Lys Leu Ala Gln Glu Phe Pro Glu Leu Leu Ala Ile Glu  
                     85                    90                    95  
 15 Asp Thr Thr Ser Leu Ser Tyr Arg His Gln Val Ala Glu Glu Leu Gly  
                     100                    105                    110  
 Lys Leu Gly Ser Ile Gln Asp Lys Ser Arg Gly Trp Trp Val His Ser  
                     115                    120                    125  
 20 Val Leu Leu Leu Glu Ala Thr Thr Phe Arg Thr Val Gly Leu Leu His  
                     130                    135                    140  
 Gln Glu Trp Trp Met Arg Pro Asp Asp Pro Ala Asp Ala Asp Glu Lys  
                     145                    150                    155                    160  
 Glu Ser Gly Lys Trp Leu Ala Ala Ala Ala Thr Ser Arg Leu Arg Met  
                     165                    170                    175  
 25 Gly Ser Met Met Ser Asn Val Ile Ala Val Cys Asp Arg Glu Ala Asp  
                     180                    185                    190  
 Ile His Ala Tyr Leu Gln Asp Arg Leu Ala His Asn Glu Arg Phe Val  
                     195                    200                    205  
 30 Val Arg Ser Lys His Pro Arg Lys Asp Val Glu Ser Gly Leu Tyr Leu  
                     210                    215                    220  
 Ile Asp His Leu Lys Asn Gln Pro Glu Leu Gly Gly Tyr Gln Ile Ser  
                     225                    230                    235                    240  
 Ile Pro Gln Lys Gly Val Val Asp Lys Arg Gly Lys Arg Lys Asn Arg  
                     245                    250                    255  
 35 Pro Ala Arg Lys Ala Ser Leu Ser Leu Arg Ser Gly Arg Ile Thr Leu  
                     260                    265                    270  
 Lys Gln Gly Asn Ile Thr Leu Asn Ala Val Leu Ala Glu Glu Ile Asn  
                     275                    280                    285  
 40 Pro Pro Lys Gly Glu Thr Pro Leu Lys Trp Leu Leu Leu Thr Gly Glu  
                     290                    295                    300  
 Pro Val Glu Ser Leu Ala Gln Ala Leu Arg Val Ile Asp Ile Tyr Thr  
                     305                    310                    315                    320  
 His Arg Trp Arg Ile Glu Glu Phe His Lys Ala Trp Lys Thr Gly Ala  
                     325                    330                    335  
 45 Gly Ala Glu Arg Gln Arg Met Glu Glu Pro Asp Asn Leu Glu Arg Met  
                     340                    345                    350  
 Val Ser Ile Leu Ser Phe Val Ala Val Arg Leu Leu Gln Leu Arg Glu  
                     355                    360                    365

5 Ser Phe Thr Pro Pro Gln Ala Leu Arg Ala Gln Gly Leu Leu Lys Glu  
370 375 380

Ala Glu His Val Glu Ser Gln Ser Ala Glu Thr Val Leu Thr Pro Asp  
385 390 395 400

10 Glu Cys Gln Leu Leu Gly Tyr Leu Asp Lys Gly Lys Arg Lys Arg Lys  
405 410 415

Glu Lys Ala Gly Ser Leu Gln Trp Ala Tyr Met Ala Ile Ala Arg Leu  
420 425 430

Gly Gly Phe Met Asp Ser Lys Arg Thr Gly Ile Ala Ser Trp Gly Ala  
435 440 445

15 Leu Trp Glu Gly Trp Glu Ala Leu Gln Ser Lys Leu Asp Gly Phe Leu  
450 455 460

Ala Ala Lys Asp Leu Met Ala Gln Gly Ile Lys Ile \*

465 470 475

## (2) INFORMATION FOR SEQ ID NO:3:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5838 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: circular
- 25 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Plasmid DNA"
- (vii) IMMEDIATE SOURCE:  
(B) CLONE: pRZTL1
- 30 (ix) FEATURE:  
(A) NAME/KEY: insertion\_seq  
(B) LOCATION: 1..19
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 77..1267  
35 (D) OTHER INFORMATION: /function= "tetracycline resistance"
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: complement (2301..2960)  
40 (D) OTHER INFORMATION: /function= "chloramphenicol resistance"
- (ix) FEATURE:  
(A) NAME/KEY: insertion\_seq  
(B) LOCATION: 4564..4582
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
45 (B) LOCATION: 4715..5530  
(D) OTHER INFORMATION: /function= "kanamycin resistance"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGACTCTTA TACACAAGTA AGCTTTAATG CGGTAGTTTA TCACAGTTAA ATTGCTAACG 60

CAGTCAGGCA CCGTGT ATG AAA TCT AAC AAT GCG CTC ATC GTC ATC CTC 109

50 Met Lys Ser Asn Asn Ala Leu Ile Val Ile Leu  
480 485

5	GGC ACC GTC ACC CTG GAT GCT GTA GGC ATA GGC TTG GTT ATG CCG GTA Gly Thr Val Thr Leu Asp Ala Val Gly Ile Gly Leu Val Met Pro Val 490 495 500	157
10	CTG CCG GGC CTC TTG CGG GAT ATC GTC CAT TCC GAC AGC ATC GCC AGT Leu Pro Gly Leu Leu Arg Asp Ile Val His Ser Asp Ser Ile Ala Ser 505 510 515 520	205
	CAC TAT GGC GTG CTG CTA GCG CTA TAT GCG TTG ATG CAA TTT CTA TGC His Tyr Gly Val Leu Leu Ala Leu Tyr Ala Leu Met Gln Phe Leu Cys 525 530 535	253
15	GCA CCC GTT CTC GGA GCA CTG TCC GAC CGC TTT GGC CGC CGC CCA GTC Ala Pro Val Leu Gly Ala Leu Ser Asp Arg Phe Gly Arg Arg Pro Val 540 545 550	301
	CTG CTC GCT TCG CTA CTT GGA GCC ACT ATC GAC TAC GCG ATC ATG GCG Leu Leu Ala Ser Leu Leu Gly Ala Thr Ile Asp Tyr Ala Ile Met Ala 555 560 565	349
20	ACC ACA CCC GTC CTG TGG ATC CTC TAC GCC GGA CGC ATC GTG GCC GGC Thr Thr Pro Val Leu Trp Ile Leu Tyr Ala Gly Arg Ile Val Ala Gly 570 575 580	397
25	ATC ACC GGC GCC ACA GGT GCG GTT GCT GGC GCC TAT ATC GCC GAC ATC Ile Thr Gly Ala Thr Gly Ala Val Ala Gly Ala Tyr Ile Ala Asp Ile 585 590 595 600	445
	ACC GAT GGG GAA GAT CGG GCT CGC CAC TTC GGG CTC ATG AGC GCT TGT Thr Asp Gly Glu Asp Arg Ala Arg His Phe Gly Leu Met Ser Ala Cys 605 610 615	493
30	TTC GGC GTG GGT ATG GTG GCA GGC CCC GTG GCC GGG GGA CTG TTG GGC Phe Gly Val Gly Met Val Ala Gly Pro Val Ala Gly Gly Leu Leu Gly 620 625 630	541
	GCC ATC TCC TTG CAT GCA CCA TTC CTT GCG GCG GCG GTG CTC AAC GGC Ala Ile Ser Leu His Ala Pro Phe Leu Ala Ala Ala Val Leu Asn Gly 635 640 645	589
35	CTC AAC CTA CTA CTG GGC TGC TTC CTA ATG CAG GAG TCG CAT AAG GGA Leu Asn Leu Leu Leu Gly Cys Phe Leu Met Gln Glu Ser His Lys Gly 650 655 660	637
40	GAG CGT CGA CCG ATG CCC TTG AGA GCC TTC AAC CCA GTC AGC TCC TTC Glu Arg Arg Pro Met Pro Leu Arg Ala Phe Asn Pro Val Ser Ser Phe 665 670 675 680	685
	CGG TGG GCG CGG GGC ATG ACT ATC GTC GCC GCA CTT ATG ACT GTC TTC Arg Trp Ala Arg Gly Met Thr Ile Val Ala Ala Leu Met Thr Val Phe 685 690 695	733
45	TTT ATC ATG CAA CTC GTA GGA CAG GTG CCG GCA GCG CTC TGG GTC ATT Phe Ile Met Gln Leu Val Gly Gln Val Pro Ala Ala Leu Trp Val Ile 700 705 710	781
	TTC GGC GAG GAC CGC TTT CGC TGG AGC GCG ACG ATG ATC GGC CTG TCG Phe Gly Glu Asp Arg Phe Arg Trp Ser Ala Thr Met Ile Gly Leu Ser 715 720 725	829
50	CTT GCG GTA TTC GGA ATC TTG CAC GCC CTC GCT CAA GCC TTC GTC ACT Leu Ala Val Phe Gly Ile Leu His Ala Leu Ala Gln Ala Phe Val Thr 730 735 740	877
55	GGT CCC GCC ACC AAA CGT TTC GGC GAG AAG CAG GCC ATT ATC GCC GGC Gly Pro Ala Thr Lys Arg Phe Gly Glu Lys Gln Ala Ile Ile Ala Gly 745 750 755 760	925

5	ATG GCG GCC GAC GCG CTG GGC TAC GTC TTG CTG GCG TTC GCG ACG CGA Met Ala Ala Asp Ala Leu Gly Tyr Val Leu Leu Ala Phe Ala Thr Arg 765 770 775	973
10	GGC TGG ATG GCC TTC CCC ATT ATG ATT CTT CTC GCT TCC GGC GGC ATC Gly Trp Met Ala Phe Pro Ile Met Ile Leu Leu Ala Ser Gly Gly Ile 780 785 790	1021
	GGG ATG CCC GCG TTG CAG GCC ATG CTG TCC AGG CAG GTA GAT GAC GAC Gly Met Pro Ala Leu Gln Ala Met Leu Ser Arg Gln Val Asp Asp Asp 795 800 805	1069
15	CAT CAG GGA CAG CTT CAA GGA TCG CTC GCG GCT CTT ACC AGC CTA ACT His Gln Gly Gln Leu Gln Gly Ser Leu Ala Ala Leu Thr Ser Leu Thr 810 815 820	1117
	TCG ATC ACT GGA CCG CTG ATC GTC ACG GCG ATT TAT GCC GCC TCG GCG Ser Ile Thr Gly Pro Leu Ile Val Thr Ala Ile Tyr Ala Ala Ser Ala 825 830 835 840	1165
20	AGC ACA TGG AAC GGG TTG GCA TGG ATT GTA GGC GCC GCC CTA TAC CTT Ser Thr Trp Asn Gly Leu Ala Trp Ile Val Gly Ala Ala Leu Tyr Leu 845 850 855	1213
25	GTC TGC CTC CCC GCG TTG CGT CGC GGT GCA TGG AGC CGG GCC ACC TCG Val Cys Leu Pro Ala Leu Arg Arg Gly Ala Trp Ser Arg Ala Thr Ser 860 865 870	1261
	ACC TGA ATGGAAGCCG GCGGCACCTC GCTAACGGAT TCACCACTCC AAGAATTGGA Thr *	1317
30	GCCAAATCAAT TCTTGCGGAG AACTGTGAAT GCGCAAACCA ACCCTTGGCA GAACATATCC ATCGCGTCCG CCATCTCCAG CAGCCGCACG CGGCGCATCT CGGGCAGCGT TGGGTCCTGG CCACGGGTGC GCATGATCGT GCTCCTGTCTG TTGAGGACCC GGCTAGGCTG GCGGGGTTGC CTTACTGGTT AGCAGAATGA ATCACCATA CGCGAGCGAA CGTGAAGCGA CTGCTGCTGC AAAACGTCTG CGACCTGAGC AACAACATGA ATGGTCTTCG GTTTCCTGTG TTCGTAAAGT CTGGAACGC GGAAGTCCCC TACGTGCTGC TGAAGTTGCC CGCAACAGAG AGTGAACCA ACCGGTGATA CCACGATACT ATGACTGAGA GTCAACGCCA TGAGCGGCCT CATTTCTTAT TCTGAGTTAC AACAGTCCGC ACCGCTGTCC GGTAGCTCCT TCCGGTGGGC GCGGGGCATG ACTATCGTCG CCGCACTTAT GACTGTCTTC TTTATCATGC AACTCGTAGG ACAGGTGCCG GCAGCGCCCA ACAGTCCCC GGCCACGGG CCTGCCACCA TACCCACGCC GAAACAAGCG CCCTGCACCA TTATGTTCCG GATCTGCATC GCAGGATGCT GCTGGCTACC CTGTGGAACA CCTACATCTG TATTAACGAA GCGCTAACCG TTTTATCAG GCTCTGGGAG GCAGAATAAA TGATCATATC GTCAATTATT ACCTCCACGG GGAGAGCCTG AGCAAACTGG CCTCAGGCAT TTGAGAAGCA CACGGTCACA CTGCTTCCGG TAGTCAATAA ACCGGTAAAC CAGCAATAGA CATAAGCGGC TATTTAACGA CCCTGCCCTG AACCGACGAC CGGGTCGAAT TTGCTTTCGA ATTTCTGCCA TTCATCCGCT TATTATCAAT TATTCAGGCG TAGCACCAGG CGTTTAAGGG CACCAATAAC TGCCTTAAAA AAATTACGCC CCGCCCTGCC ACTCATCGCA GTACTGTTGT AATTCATTAA GCATTCTGCC GACATGGAAG CCATCACAGA CGGCATGATG AACCTGAATC	1377 1437 1497 1557 1617 1677 1737 1797 1857 1917 1977 2037 2097 2157 2217 2277 2337 2397

5	GCCAGCGGCA TCAGCACCTT GTCGCCTTGC GTATAATATT TGCCCATGGT GAAAACGGGG	2457
	GCGAAGAAGT TGTCCATATT GGCCACGTTT AAATCAAAAC TGGTGAAACT CACCCAGGGA	2517
	TTGGCTGAGA CGAAAAACAT ATTCTCAATA AACCCCTTAG GGAAATAGGC CAGGTTTTCA	2577
	CCGTAACACG CCACATCTTG CGAATATATG TGTAGAAACT GCCGGAAATC GTCGTGGTAT	2637
	TCACTCCAGA GCGATGAAAA CGTTTCAGTT TGCTCATGGA AAACGGTGTA ACAAGGGTGA	2697
10	AACTATCCC ATATCACCAG CTCACCGTCT TTCATTGCCA TACGGAATTC CGGATGAGCA	2757
	TTCATCAGGC GGGCAAGAAT GTGAATAAAG GCCGGATAAA ACTTGTGCTT ATTTTTCTTT	2817
	ACGGTCTTTA AAAAGGCCGT AATATCCAGC TGAACGGTCT GGTATAGGT ACATTGAGCA	2877
	ACTGACTGAA ATGCCTCAA ATGTTCTTTA CGATGCCATT GGGATATATC AACGGTGGA	2937
	TATCCAGTGA TTTTTTCTC CATTTTAGCT TCCTTAGCTC CTGAAAATCT CGATAACTCA	2997
15	AAAAATACGC CCGGTAGTGA TCTTATTTCA TTATGGTGAA AGTTGGAACC TCTTACGTGC	3057
	CGATCAACGT CTCATTTTCG CCAAAAGTTG GCCCAGGGCT TCCCGGTATC AACAGGGACA	3117
	CCAGGATTTA TTTATTCTGC GAAGTGATCT TCCGTCACAG GTATTTATTC GGCACAAAGT	3177
	GCGTCGGGTG ATGCTGCCAA CTTACTGATT TAGTGATGA TGGTGTTTTT GAGGTGCTCC	3237
	AGTGGCTTCT GTTCTATCA GCTGTCCCTC CTGTTAGCT ACTGACGGGG TGGTGCCTAA	3297
20	CGGCAAAAGC ACCGCCGGAC ATCAGCGCTA GCGGAGTGTA TACTGGCTTA CTATGTTGGC	3357
	ACTGATGAGG GTGTCAGTGA AGTGCTTCAT GTGGCAGGAG AAAAAAGGCT GCACCGGTGC	3417
	GTCAGCAGAA TATGTGATAC AGGATATATT CCGCTTCCTC GCTCACTGAC TCGCTACGCT	3477
	CGGTGCTTCG ACTGCGGCGA GCGGAAATGG CTTACGAACG GGGCGGAGAT TTCCTGGAAG	3537
	ATGCCAGGAA GATACTTAAC AGGGAAGTGA GAGGGCCGCG GCAAAGCCGT TTTTCCATAG	3597
25	GCTCCGCCCC CCTGACAAGC ATCAGCAAAT CTGACGCTCA AATCAGTGGT GGCAGAAACCC	3657
	GACAGGACTA TAAAGATACC AGGCGTTTCC CCTGGCGGCT CCCTCGTGCG CTCTCCTGTT	3717
	CCTGCCTTTC GGTTTACCGG TGTCATTCCG CTGTTATGGC CGCGTTTGTC TCATTCCACG	3777
	CCTGACACTC AGTTCCGGGT AGGCAGTTCG CTCCAAGCTG GACTGTATGC ACGAACCCCC	3837
	CGTTCAGTCC GACCGCTGCG CTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGAAAG	3897
30	ACATGCAAAA GCACCACTGG CAGCAGCCAC TGGTAATTGA TTAGAGGAG TTAGTCTTGA	3957
	AGTCATGCGC CGGTTAAGGC TAACTGAAA GGACAAGTTT TGGTGACTGC GCTCCTCCAA	4017
	GCCAGTTACC TCGGTTCAAA GAGTTGGTAG CTCAGAGAAC CTTGAAAAA CCGCCCTGCA	4077
	AGGCGGTTTT TTCGTTTTCA GAGCAAGAGA TTACGCGCAG ACCAAAACGA TCTCAAGAAG	4137
	ATCATCTTAT TAATCAGATA AAATATTTCT AGAGGTGAAC CATCACCCTA ATCAAGTTTT	4197
35	TTGGGGTCGA GGTGCCGTAA AGCACTAAAT CGGAACCCTA AAGGGATGCC CCGATTTAGA	4257
	GCTTGACGGG GAAAGCCGGC GAACGTGGCG AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG	4317
	GGCGTAGGG CGCTGGCAAG TGAGCGGTC ACGCTGCGCG TAACCACCAC ACCCGCCGCG	4377
	CTTAATGCGC CGCTACAGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA	4437



5	TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCCGA	4497
	TTAAGTTGGG TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGCC	4557
	AAGCTTACTT GTGTATAAGA GTCAGTCGAC CTGCAGGGGG GGGGGGGAAA GCCACGTTGT	4617
	GTCTCAAAAT CTCTGATGTT ACATTGCACA AGATAAAAAT ATATCATCAT GAACAATAAA	4677
10	ACTGTCTGCT TACATAAACA GTAATACAAG GGGTGTT ATG AGC CAT ATT CAA CGG Met Ser His Ile Gln Arg 1 5	4732
	GAA ACG TCT TGC TCG AGG CCG CGA TTA AAT TCC AAC ATG GAT GCT GAT Glu Thr Ser Cys Ser Arg Pro Arg Leu Asn Ser Asn Met Asp Ala Asp 10 15 20	4780
15	TTA TAT GGG TAT AAA TGG GCT CGC GAT AAT GTC GGG CAA TCA GGT GCG Leu Tyr Gly Tyr Lys Trp Ala Arg Asp Asn Val Gly Gln Ser Gly Ala 25 30 35	4828
	ACA ATC TAT CGA TTG TAT GGG AAG CCC GAT GCG CCA GAG TTG TTT CTG Thr Ile Tyr Arg Leu Tyr Gly Lys Pro Asp Ala Pro Glu Leu Phe Leu 40 45 50	4876
20	AAA CAT GGC AAA GGT AGC GTT GCC AAT GAT GTT ACA GAT GAG ATG GTC Lys His Gly Lys Gly Ser Val Ala Asn Asp Val Thr Asp Glu Met Val 55 60 65 70	4924
25	AGA CTA AAC TGG CTG ACG GAA TTT ATG CCT CTT CCG ACC ATC AAG CAT Arg Leu Asn Trp Leu Thr Glu Phe Met Pro Leu Pro Thr Ile Lys His 75 80 85	4972
	TTT ATC CGT ACT CCT GAT GAT GCA TGG TTA CTC ACC ACT GCG ATC CCC Phe Ile Arg Thr Pro Asp Asp Ala Trp Leu Leu Thr Thr Ala Ile Pro 90 95 100	5020
30	GGG AAA ACA GCA TTC CAG GTA TTA GAA GAA TAT CCT GAT TCA GGT GAA Gly Lys Thr Ala Phe Gln Val Leu Glu Glu Tyr Pro Asp Ser Gly Glu 105 110 115	5068
	AAT ATT GTT GAT GCG CTG GCA GTG TTC CTG CGC CGG TTG CAT TCG ATT Asn Ile Val Asp Ala Leu Ala Val Phe Leu Arg Arg Leu His Ser Ile 120 125 130	5116
35	CCT GTT TGT AAT TGT CCT TTT AAC AGC GAT CGC GTA TTT CGT CTC GCT Pro Val Cys Asn Cys Pro Phe Asn Ser Asp Arg Val Phe Arg Leu Ala 135 140 145 150	5164
40	CAG GCG CAA TCA CGA ATG AAT AAC GGT TTG GTT GAT GCG AGT GAT TTT Gln Ala Gln Ser Arg Met Asn Asn Gly Leu Val Asp Ala Ser Asp Phe 155 160 165	5212
	GAT GAC GAG CGT AAT GGC TGG CCT GTT GAA CAA GTC TGG AAA GAA ATG Asp Asp Glu Arg Asn Gly Trp Pro Val Glu Gln Val Trp Lys Glu Met 170 175 180	5260
45	CAT AAG CTT TTG CCA TTC TCA CCG GAT TCA GTC GTC ACT CAT GGT GAT His Lys Leu Leu Pro Phe Ser Pro Asp Ser Val Val Thr His Gly Asp 185 190 195	5308
	TTC TCA CTT GAT AAC CTT ATT TTT GAC GAG GGG AAA TTA ATA GGT TGT Phe Ser Leu Asp Asn Leu Ile Phe Asp Glu Gly Lys Leu Ile Gly Cys 200 205 210	5356
50	ATT GAT GTT GGA CGA GTC GGA ATC GCA GAC CGA TAC CAG GAT CTT GCC Ile Asp Val Gly Arg Val Gly Ile Ala Asp Arg Tyr Gln Asp Leu Ala 215 220 225 230	5404

5 ATC CTA TGG AAC TGC CTC GGT GAG TTT TCT CCT TCA TTA CAG AAA CGG 5452  
 Ile Leu Trp Asn Cys Leu Gly Glu Phe Ser Pro Ser Leu Gln Lys Arg  
 235 240 245

CTT TTT CAA AAA TAT GGT ATT GAT AAT CCT GAT ATG AAT AAA TTG CAG 5500  
 Leu Phe Gln Lys Tyr Gly Ile Asp Asn Pro Asp Met Asn Lys Leu Gln  
 250 255 260

10 TTT CAT TTG ATG CTC GAT GAG TTT TTC TAA TCAGAATTGG TTAATTGGTT 5550  
 Phe His Leu Met Leu Asp Glu Phe Phe \*  
 265 270

GTAACACTGG CAGAGCATTG CGCTGACTTG ACGGGACGGC GGCTTTGTTG AATAAATCGA 5610

15 ACTTTTGCTG AGTTGAAGGA TCAGATCAGC CATCTTCCCG ACAACGCAGA CCGTTCCGTG 5670

GCAAAGCAAA AGTTCAAAAT CACCAACTGG TCCACCTACA ACAAAGCTCT CATCAACCGT 5730

GGCTCCCTCA CTTTCTGGCT GGATGATGGG GCGATTGAGG CCTGGTATGA GTCAGCAACA 5790

CCTTCTTCAC GAGGCAGACC TCAGCGCCCC CCCCCCCTG CAGGTCGA 5838

## (2) INFORMATION FOR SEQ ID NO:4:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 397 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Ser Asn Asn Ala Leu Ile Val Ile Leu Gly Thr Val Thr Leu  
 1 5 10 15

Asp Ala Val Gly Ile Gly Leu Val Met Pro Val Leu Pro Gly Leu Leu  
 20 25 30

30 Arg Asp Ile Val His Ser Asp Ser Ile Ala Ser His Tyr Gly Val Leu  
 35 40 45

Leu Ala Leu Tyr Ala Leu Met Gln Phe Leu Cys Ala Pro Val Leu Gly  
 50 55 60

35 Ala Leu Ser Asp Arg Phe Gly Arg Arg Pro Val Leu Leu Ala Ser Leu  
 65 70 75 80

Leu Gly Ala Thr Ile Asp Tyr Ala Ile Met Ala Thr Thr Pro Val Leu  
 85 90 95

Trp Ile Leu Tyr Ala Gly Arg Ile Val Ala Gly Ile Thr Gly Ala Thr  
 100 105 110

40 Gly Ala Val Ala Gly Ala Tyr Ile Ala Asp Ile Thr Asp Gly Glu Asp  
 115 120 125

Arg Ala Arg His Phe Gly Leu Met Ser Ala Cys Phe Gly Val Gly Met  
 130 135 140

45 Val Ala Gly Pro Val Ala Gly Gly Leu Leu Gly Ala Ile Ser Leu His  
 145 150 155 160

Ala Pro Phe Leu Ala Ala Val Leu Asn Gly Leu Asn Leu Leu Leu  
 165 170 175

5 Gly Cys Phe Leu Met Gln Glu Ser His Lys Gly Glu Arg Arg Pro Met  
180 185 190

Pro Leu Arg Ala Phe Asn Pro Val Ser Ser Phe Arg Trp Ala Arg Gly  
195 200 205

10 Met Thr Ile Val Ala Ala Leu Met Thr Val Phe Phe Ile Met Gln Leu  
210 215 220

Val Gly Gln Val Pro Ala Ala Leu Trp Val Ile Phe Gly Glu Asp Arg  
225 230 235 240

Phe Arg Trp Ser Ala Thr Met Ile Gly Leu Ser Leu Ala Val Phe Gly  
245 250 255

15 Ile Leu His Ala Leu Ala Gln Ala Phe Val Thr Gly Pro Ala Thr Lys  
260 265 270

Arg Phe Gly Glu Lys Gln Ala Ile Ile Ala Gly Met Ala Ala Asp Ala  
275 280 285

20 Leu Gly Tyr Val Leu Leu Ala Phe Ala Thr Arg Gly Trp Met Ala Phe  
290 295 300

Pro Ile Met Ile Leu Leu Ala Ser Gly Gly Ile Gly Met Pro Ala Leu  
305 310 315 320

Gln Ala Met Leu Ser Arg Gln Val Asp Asp Asp His Gln Gly Gln Leu  
325 330 335

25 Gln Gly Ser Leu Ala Ala Leu Thr Ser Leu Thr Ser Ile Thr Gly Pro  
340 345 350

Leu Ile Val Thr Ala Ile Tyr Ala Ala Ser Ala Ser Thr Trp Asn Gly  
355 360 365

30 Leu Ala Trp Ile Val Gly Ala Ala Leu Tyr Leu Val Cys Leu Pro Ala  
370 375 380

Leu Arg Arg Gly Ala Trp Ser Arg Ala Thr Ser Thr \*

385 390 395

## (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
35 (A) LENGTH: 220 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

40 Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr Val Asp Ile Ser Gln Trp  
1 5 10 15

His Arg Lys Glu His Phe Glu Ala Phe Gln Ser Val Ala Gln Cys Thr  
20 25 30

45 Tyr Asn Gln Thr Val Gln Leu Asp Ile Thr Ala Phe Leu Lys Thr Val  
35 40 45

Lys Lys Asn Lys His Lys Phe Tyr Pro Ala Phe Ile His Ile Leu Ala  
50 55 60

Arg Leu Met Asn Ala His Pro Glu Phe Arg Met Ala Met Lys Asp Gly  
65 70 75 80

5 Glu Leu Val Ile Trp Asp Ser Val His Pro Cys Tyr Thr Val Phe His  
85 90 95

Glu Gln Thr Glu Thr Phe Ser Ser Leu Trp Ser Glu Tyr His Asp Asp  
100 105 110

10 Phe Arg Gln Phe Leu His Ile Tyr Ser Gln Asp Val Ala Cys Tyr Gly  
115 120 125

Glu Asn Leu Ala Tyr Phe Pro Lys Gly Phe Ile Glu Asn Met Phe Phe  
130 135 140

Val Ser Ala Asn Pro Trp Val Ser Phe Thr Ser Phe Asp Leu Asn Val  
145 150 155 160

15 Ala Asn Met Asp Asn Phe Phe Ala Pro Val Phe Thr Met Gly Lys Tyr  
165 170 175

Tyr Thr Gln Gly Asp Lys Val Leu Met Pro Leu Ala Ile Gln Val His  
180 185 190

His Ala Val Cys Asp Gly Phe His Val Gly Arg Met Leu Asn Glu Leu  
195 200 205

20 Gln Gln Tyr Cys Asp Glu Trp Gln Gly Gly Ala \*  
210 215 220

## (2) INFORMATION FOR SEQ ID NO:6:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 272 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

30 Met Ser His Ile Gln Arg Glu Thr Ser Cys Ser Arg Pro Arg Leu Asn  
1 5 10 15

Ser Asn Met Asp Ala Asp Leu Tyr Gly Tyr Lys Trp Ala Arg Asp Asn  
20 25 30

35 Val Gly Gln Ser Gly Ala Thr Ile Tyr Arg Leu Tyr Gly Lys Pro Asp  
35 40 45

Ala Pro Glu Leu Phe Leu Lys His Gly Lys Gly Ser Val Ala Asn Asp  
50 55 60

Val Thr Asp Glu Met Val Arg Leu Asn Trp Leu Thr Glu Phe Met Pro  
65 70 75 80

40 Leu Pro Thr Ile Lys His Phe Ile Arg Thr Pro Asp Asp Ala Trp Leu  
85 90 95

Leu Thr Thr Ala Ile Pro Gly Lys Thr Ala Phe Gln Val Leu Glu Glu  
100 105 110

45 Tyr Pro Asp Ser Gly Glu Asn Ile Val Asp Ala Leu Ala Val Phe Leu  
115 120 125

Arg Arg Leu His Ser Ile Pro Val Cys Asn Cys Pro Phe Asn Ser Asp  
130 135 140

Arg Val Phe Arg Leu Ala Gln Ala Gln Ser Arg Met Asn Asn Gly Leu  
145 150 155 160

5 Val Asp Ala Ser Asp Phe Asp Asp Glu Arg Asn Gly Trp Pro Val Glu  
165 170 175  
Gln Val Trp Lys Glu Met His Lys Leu Leu Pro Phe Ser Pro Asp Ser  
180 185 190  
10 Val Val Thr His Gly Asp Phe Ser Leu Asp Asn Leu Ile Phe Asp Glu  
195 200 205  
Gly Lys Leu Ile Gly Cys Ile Asp Val Gly Arg Val Gly Ile Ala Asp  
210 215 220  
Arg Tyr Gln Asp Leu Ala Ile Leu Trp Asn Cys Leu Gly Glu Phe Ser  
225 230 235 240  
15 Pro Ser Leu Gln Lys Arg Leu Phe Gln Lys Tyr Gly Ile Asp Asn Pro  
245 250 255  
Asp Met Asn Lys Leu Gln Phe His Leu Met Leu Asp Glu Phe Phe \*
260 265 270

## (2) INFORMATION FOR SEQ ID NO:7:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Tn5 wild type outside end"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGACTCTTA TACACAAGT

19

## (2) INFORMATION FOR SEQ ID NO:8:

- 30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Tn5 mutant outside end"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGTCTCTTA TACACATCT

19

## (2) INFORMATION FOR SEQ ID NO:9:

- 40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 45 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Tn5 mutant outside end"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTCTCTTA TACAGATCT

19

## 5 (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
10 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Tn5 wild type inside end"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGTCTCTTG ATCAGATCT

19

## 15 (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19182 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
20 (D) TOPOLOGY: circular

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Plasmid pRZ4196"

- (ix) FEATURE:  
(A) NAME/KEY: repeat\_unit  
25 (B) LOCATION: 94..112  
(D) OTHER INFORMATION: /note= "Wild type OE sequence"

- (ix) FEATURE:  
(A) NAME/KEY: repeat\_unit  
30 (B) LOCATION: 12184..12225  
(D) OTHER INFORMATION: /note= "Cassette IE"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTCCTGTAAC AATAGCAATA CCCCAAATAC CTAATGTAGT TCCAGCAAGC AAGCTAAAAA	60
GTAAAGCAAC AACATAACTC ACCCCTGCAT CTGCTGACTC TTATACACAA GTAGCGTCCC	120
GGGATCGGGA TCCCGTCGTT TTACAACGTC GTGACTGGGA AAACCCTGGC GTTACCCAAC	180
35 TTAATCGCCT TGCAGCACAT CCCCTTTTCG CCAGCTGGCG TAATAGCGAA GAGGCCCGCA	240
CCGATCGCCC TTCCCAACAG TTGCGCAGCC TGAATGGCGA ATGGCGCTTT GCCTGGTTTC	300
CGGCACCAGA AGCGGTGCCG GAAAGCTGGC TGGAGTGCGA TCTTCCTGAG GCCGATACTG	360
TCGTCGTCCC CTCAAACCTGG CAGATGCACG GTTACGATGC GCCCATCTAC ACCAACGTAA	420
CCTATCCCAT TACGGTCAAT CCGCCGTTTG TTCCCACGGA GAATCCGACG GGTTGTTACT	480
40 CGCTCACATT TAATGTTGAT GAAAGCTGGC TACAGGAAGG CCAGACGCGA ATTATTTTGT	540
ATGGCGTTAA CTCGGCGTTT CATCTGTGGT GCAACGGGCG CTGGGTCGGT TACGGCCAGG	600
ACAGTCGTTT GCCGTCTGAA TTTGACCTGA GCGCATTTTT ACGCGCCGGA GAAAACCGCC	660
TCGCGGTGAT GGTGCTGCGT TGGAGTGACG GCAGTTATCT GGAAGATCAG GATATGTGGC	720
GGATGAGCGG CATTTTCCGT GACGTCTCGT TGCTGCATAA ACCGACTACA CAAATCAGCG	780
45 ATTTCCATGT TGCCACTCGC TTTAATGATG ATTTAGCCG CGCTGTACTG GAGGCTGAAG	840
TTCAGATGTG CGGCGAGTTG CGTGACTACC TACGGGTAAC AGTTTCTTTA TGGCAGGGTG	900

5	AAACGCAGGT CGCCAGCGGC ACCGCGCCTT TCGGCGGTGA AATTATCGAT GAGCGTGGTG	960
	GTTATGCCGA TCGCGTCACA CTACGTCTGA ACGTCGAAAA CCCGAAACTG TGGAGCGCCG	1020
	AAATCCCGAA TCTCTATCGT GCGGTGGTTG AACTGCACAC CGCCGACGGC ACGCTGATTG	1080
	AAGCAGAAGC CTGCGATGTC GGTTCGCG AGGTGCGGAT TGAAAATGGT CTGCTGCTGC	1140
	TGAACGGCAA GCCGTTGCTG ATTTCGAGGCG TTAACCGTCA CGAGCATCAT CCTCTGCATG	1200
10	GTCAGGTCAT GGATGAGCAG ACGATGGTGC AGGATATCCT GCTGATGAAG CAGAACAAC	1260
	TTAACGCCGT GCGCTGTTTCG CATTATCCGA ACCATCCGCT GTGGTACACG CTGTGCGACC	1320
	GCTACGGCCT GTATGTGGTG GATGAAGCCA ATATTGAAAC CCACGGCATG GTGCCAATGA	1380
	ATCGTCTGAC CGATGATCCG CGCTGGCTAC CGGCGATGAG CGAACGCGTA ACGCGAATGG	1440
	TGCAGCGCGA TCGTAATCAC CCGAGTGTGA TCATCTGGTC GCTGGGGAAT GAATCAGGCC	1500
15	ACGGCGCTAA TCACGACGCG CTGTATCGCT GGATCAAATC TGTCGATCCT TCCCCCCCGG	1560
	TGCAGTATGA AGGCGGCGGA GCCGACACCA CGGCCACCGA TATTATTTGC CCGATGTACG	1620
	CGCGCGTGGA TGAAGACCAG CCCTTCCCGG CTGTGCCGAA ATGGTCCATC AAAAAATGGC	1680
	TTTCGCTACC TGGAGAGACG CGCCCGCTGA TCCTTTGCGA ATACGCCCAC GCGATGGGTA	1740
	ACAGTCTTGG CGGTTTCGCT AAATACTGGC AGGCGTTTCG TCAGTATCCC CGTTTACAGG	1800
20	GCGGCTTCGT CTGGGACTGG GTGGATCAGT CGCTGATTAA ATATGATGAA AACGGCAACC	1860
	CGTGGTCGGC TTACGGCGGT GATTTTGGCG ATACGCCGAA CGATCGCCAG TTCTGTATGA	1920
	ACGGTCTGGT CTTTGCCGAC CGCACGCCGC ATCCAGCGCT GACGGAAGCA AAACACCAGC	1980
	AGCAGTTTTT CCAGTTCCGT TTATCCGGGC AAACCATCGA AGTGACCAGC GAATACCTGT	2040
	TCCGTCATAG CGATAACGAG CTCCTGCACT GGATGGTGGC GCTGGATGGT AAGCCGCTGG	2100
25	CAAGCGGTGA AGTGCCTCTG GATGTCGCTC CACAAGGTAA ACAGTTGATT GAACTGCCTG	2160
	AACTACCGCA GCCGGAGAGC GCCGGGCAAC TCTGGCTCAC AGTACGCGTA GTGCAACCGA	2220
	ACGCGACCGC ATGGTCAGAA GCCGGGCACA TCAGCGCCTG GCAGCAGTGG CGTCTGGCGG	2280
	AAAACCTCAG TGTGACGCTC CCCGCCGCGT CCCACGCCAT CCCGCATCTG ACCACCAGCG	2340
	AAATGGATTT TTGCATCGAG CTGGGTAATA AGCGTTGGCA ATTTAACCGC CAGTCAGGCT	2400
30	TTCTTTCACA GATGTGGATT GGCGATAAAA AACAACGCT GACGCCGCTG CGCGATCAGT	2460
	TCACCCGTGC ACCGCTGGAT AACGACATTG GCGTAAGTGA AGCGACCCGC ATTGACCCTA	2520
	ACGCCTGGGT CGAACGCTGG AAGGCGGCGG GCCATTACCA GGCCGAAGCA GCGTTGTTGC	2580
	AGTGCACGGC AGATACACTT GCTGATGCGG TGCTGATTAC GACCGCTCAC GCGTGGCAGC	2640
	ATCAGGGGAA AACCTTATTT ATCAGCCGGA AAACCTACCG GATTGATGGT AGTGGTCAAA	2700
35	TGGCGATTAC CGTTGATGTT GAAGTGGCGA GCGATACACC GCATCCGGCG CGGATTGGCC	2760
	TGAACTGCCA GCTGGCGCAG GTAGCAGAGC GGGTAAACTG GCTCGGATTA GGGCCGCAAG	2820
	AAAACATATC CGACCGCCTT ACTGCCGCCT GTTTTGACCG CTGGGATCTG CCATTGTCAG	2880
	ACATGTATAC CCCGTACGTC TTCCCGAGCG AAAACGGTCT GCGCTGCGGG ACGCGCGAAT	2940

5	TGAATTATGG CCCACACCAG TGGCGCGGCG ACTTCCAGTT CAACATCAGC CGCTACAGTC	3000
	AACAGCAACT GATGGAAACC AGCCATCGCC ATCTGCTGCA CGCGGAAGAA GGCACATGGC	3060
	TGAATATCGA CGGTTTCCAT ATGGGGATTG GTGGCGACGA CTCCTGGAGC CCGTCAGTAT	3120
	CGGCGGATTG CAGCTGAGCG CCGGTCGCTA CCATTACCAG TTGGTCTGGT GTCAAAAATA	3180
	ATAATAACCG GGCAGGCCAT GTCTGCCCGT ATTTGCGGTA AGGAAATCCA TTATGTACTA	3240
10	TTTAAAAAAC ACAAACTTTT GGATGTTCGG TTTATTCTTT TTCTTTTACT TTTTATCAT	3300
	GGGAGCCTAC TTCCCGTTTT TCCCGATTTG GCTACATGAC ATCAACCATA TCAGCAAAAG	3360
	TGATACGGGT ATTATTTTTG CCGCTATTTT TCTGTTCTCG CTATTATTCC AACCGCTGTT	3420
	TGGTCTGCTT TCTGACAAAC TCGGGCTGCG CAAATACCTG CTGTGGATTA TTACCGGCAT	3480
	GTTAGTGATG TTTGCGCCGT TCTTTATTTT TATCTTCGGG CCACTGTTAC AATACAACAT	3540
15	TTTAGTAGGA TCGATTGTTG GTGGTATTTA TCTAGGCTTT TGTTTTAACG CCGGTGCGCC	3600
	AGCAGTAGAG GCATTTATTG AGAAAGTCAG CCGTCGCAGT AATTTCGAAT TTGGTCGCGC	3660
	GCGGATGTTT GGCTGTGTTG GCTGGGCGCT GTGTGCCTCG ATTGTGCGCA TCATGTTTAC	3720
	CATCAATAAT CAGTTTGTTT TCTGGCTGGG CTCTGGCTGT GCACTCATCC TCGCCGTTTT	3780
	ACTCTTTTTT GCCAAAACGG ATGCGCCCTC TTCTGCCACG GTTGCCAATG CGGTAGGTGC	3840
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	GTTTTTGTC CAATGATGTTA TTGGCGTTTC CTGCACCTAC GATGTTTTTG ACCAACAGTT	3960
	TGCTAATTTT TTTACTTCGT TCTTTGCTAC CCGTGAACAG GGTACGCGGG TATTTGGCTA	4020
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	TAATCGCATC GGTGGGAAAA ACGCCCTGCT GCTGGCTGGC ACTATTATGT CTGTACGTAT	4140
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	GCGTTTTTCA GCGACGATT ATCTGGTCTG TTTCTGCTTC TTTAAGCAAC TGGCGATGAT	4320
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	AATGACCGAA AGAATAAGAG CAGGCAAGCT ATTTACCGAT ATGTGCGAAG GCTTACCGGA	4620
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	TGAAAAAGA GAAAGCCTGA TTAAAGAAAT GTTTGCCACG GTAGGGGAAA ACGCCTGGGT	4740
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	AAATTTCAAT TTAACCATTG TCGATGACTA CACGGTAACA ATCGGTGATA ACGTACTGAT	4860
	TGCACCCAAC GTTACTCTTT CCGTTACGGG ACACCCTGTA CACCATGAAT TGAGAAAAAA	4920
	CGGCGAGATG TACTCTTTTC CGATAACGAT TGGCAATAAC GTCTGGATCG GAAGTCATGT	4980



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	ACCAAGCCCA CCGCCGCCCA TCAGAGCCGC GGACCACAGC CCCATCACCA GTGGCGTGCG	6060
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	TAGCTTCAAA TAAAACCTAT CTATTTTATT TATCTTTCAA GCTCAATAAA AAGCCGCGGT	9000
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	GGTCCGGACC GCCGCCCGAT CTCCATCCGC TACAGGAATG GTTCCAGCCG CTTTTCCGGT	9540
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	GCGCAGCTCA TCCCCGATGG CCATCAGTGC GGCCACCACC TGAACCCGGT CACCGGAAGA	17100
	CCACTGCCCC CTGTTACCT TACGGGCTGT CTGATTCAGG TTATTTCCGA TGGCGGCCAG	17160
	CTGACGCAGT AACGGCGGTG CCAGTGTCCG CAGTTTTCCG GAACGGGCAA CCGGCTCCCC	17220

5	CAGGCAGACC CGCCGCATCC ATACCGCCAG TTGTTTACCC TCACAGCGTT CAAGTAACCG	17280
	GGCATGTTCA TCATCAGTAA CCCGTATTGT GAGCATCCTC TCGCGTTTCA TCGGTATCAT	17340
	TACCCCATGA ACAGAAATCC CCCTTACACG GAGGCATCAG TGAATAACA GGAAAAAACC	17400
	GCCCTTAACA TGGCCCGCTT TATCAGAAGC CAGACATTAA CGCTGCTGGA GAAGCTCAAC	17460
	GAACTGGACG CAGATGAACA GGCCGATATT TGTGAATCGC TTCACGACCA CGCCGATGAG	17520
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	CTCCCGGAGA CGGTCACAGC TTGTCTGTGA GCGGATGCCG GGAGCTGACA AGCCCGTCAG	17640
	GGCGCGTCAG CAGGTTTTAG CGGGTGTCTGG GCGCAGCCC TGACCCAGTC ACGTAGCGAT	17700
	AGCGGAGTGT ATACTGGCTT AACCATGCGG CATCAGTGCG GATTGTATGA AAAGTACGCC	17760
	ATGCCGGGTG TGAAATGCCG CACAGATGCG TAAGGAGAAA ATGCACGTCC AGGCGCTTTT	17820
15	CCGCTTCCTC GCTCACTGAC TCGCTACGCT CGGTCGTTCT ACTGCGGCGA GCGGTACTGA	17880
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	TGTGAGCAAA AGACCAGGAA CAGGAAGAAG GCCACGTAGC AGGCGTTTTT CCATAGGCTC	18000
	CGCCCCCTG ACGAGCATCA CAAAATAGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA	18060
	GGACTATAAA GCTACCAGGC GTTCCCCCT GGAAGCTCCC TCGTGCCTC TCCTGTTCCG	18120
20	ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GCGGCTTTCT	18180
	CATAGCTCAC GCTGTTGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT	18240
	GTGCACGAAC CCCCCGTTCA GCCCCACCGC TGCGCCTTAT CCGGTAACCTA TCGTCTTGAG	18300
	TCCAACCCGG TAAGGCACGC CTTAACGCCA CTGGCAGCAG CCACTGGTAA CCGGATTAGC	18360
	AGAGCGATGA TGGCACAAC GGTGCTACAG AGTTCTTGAA GTAGTGGCCC GACTACGGCT	18420
25	ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA	18480
	GAGTTGGTAG CTCTTGATCC GGCAACAAA CCACCGTTGG TAGCGGTGGT TTTTGTGTTT	18540
	GCAAGCAGCA GATTACGCGC AGAAAAAAG GATCTCAAGA AGATCCTTTA ATCTTTTCTA	18600
	CTGAACCGCG ATCCCCGTCA GTTTAGAAGA GGAGGATGGT GCGATGGTCC CTCCCTGAAC	18660
	ATCAGGTATA TAGTTAGCCT GACATCCAAC AAGGAGGTTT ATCGCGAATA TTCCACAAA	18720
30	AAATCTTTTC CTCATAACTC GATCCTTATA AAATGAAAAG AATATATGGC GAGGTTTAAT	18780
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	TATATGCAAT AGTGATAAGC ACTATTCTAT GTCCATATTC AAAATATGCT ATTGAATACA	18960
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35	CCGTAGCAAA ATTAAACCTA TTTATGCTAT ATAATCTACT TTGTTTGGTC CTAGCAATCC	19080
	CATTTGGATT GCTAGGACTT TTTATATCAA TAAAGAATAA TTAAATCCCT AACACCTCAT	19140
	TTATAGTATT AAGTTTATTC TTATCAATAT AGGAGCATAG AA	19182

1. A system for transposing a transposable DNA sequence *in vitro*, the system comprising:

a Tn5 transposase modified relative to a wild type Tn5 transposase, the modified transposase comprising a change relative to the wild type Tn5 transposase that causes the modified transposase to bind to Tn5 outside end repeat sequences with greater avidity than the wild type Tn5 transposase, and a change relative to the wild type Tn5 transposase that causes the modified transposase to be less likely than the wild type transposase to assume an inactive multimeric form;

a donor DNA molecule comprising the transposable DNA sequence, the DNA sequence being flanked at its 5'- and 3'-ends by the Tn5 outside end repeat sequences; and

a target DNA molecule into which the transposable element can transpose.

2. A system as claimed in Claim 1 wherein the change that causes the modified transposase to bind with greater avidity is characterized as a substitution mutation at position 54 of the wild type transposase.

3. A system as claimed in Claim 2 wherein position 54 is a lysine.

4. A system as claimed in Claim 1 wherein the change that causes the modified transposase to be less likely to assume an inactive multimeric form is characterized as a substitution mutation at position 372 of the wild type transposase.

5. A system as claimed in Claim 4 wherein position 372 is a proline.

6. A system as claimed in Claim 1 wherein the modified transposase further comprises a substitution mutation at position 56 of the wild type transposase.



7. A system as claimed in Claim 6 wherein position 56 is an alanine.

8. A system as claimed in Claim 1 wherein the donor DNA molecule is flanked at its 5'- and 3'-ends by an 18 or 19 base pair flanking DNA sequence comprising nucleotide A at position 10, nucleotide T at position 11, and nucleotide A at position 12.

9. The system as claimed in Claim 8 wherein the flanking sequence further comprises a nucleotide at position 4 selected from the group consisting of A or T.

10. The system as claimed in Claim 8 wherein the flanking sequence further comprises a nucleotide at position 15 selected from the group consisting of G or C.

11. The system as claimed in Claim 8 wherein the flanking sequence further comprises a nucleotide at position 17 selected from the group consisting of A or T.

12. The system as claimed in Claim 8 wherein the flanking sequence further comprises a nucleotide at position 18 selected from the group consisting of G or C.

13. The system as claimed in Claim 8 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACACATCT-3'.

14. The system as claimed in Claim 8 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACAGATCT-3'.

15. A Tn5 transposase modified relative to a wild type Tn5 transposase, the modified transposase comprising:

a change relative to the wild type Tn5 transposase that causes the modified transposase to bind to Tn5 outside end repeat sequences of a donor DNA with greater avidity than the wild type Tn5 transposase; and

a change relative to the wild type Tn5 transposase that causes the modified transposase to be less likely than the wild type transposase to assume an inactive multimeric form.

16. A modified Tn5 transposase as claimed in Claim 15 wherein the change that causes the modified transposase to bind with greater avidity is characterized as a substitution mutation at position 54 of the wild type transposase.

17. A modified Tn5 transposase as claimed in Claim 16 wherein position 54 is a lysine.

18. A modified Tn5 transposase as claimed in Claim 15 wherein the change that causes the modified transposase to be less likely to assume an inactive multimeric form is characterized as a substitution mutation at position 372 of the wild type transposase.

19. A modified Tn5 transposase as claimed in Claim 18 wherein position 372 is a proline.

20. A modified Tn5 transposase as claimed in Claim 15 further comprising a substitution mutation at position 56 of the wild type transposase.

21. A modified Tn5 transposase as claimed in Claim 20 wherein position 56 is alanine.

22. A genetic construct comprising a nucleotide sequence that can encode a Tn5 transposase that both has greater avidity for Tn5 outside end repeats and is less likely to assume an inactive multimeric form than a wild type Tn5 transposase.

23. A genetic construct as claimed in Claim 22 comprising a nucleotide sequence that encodes a lysine residue at amino acid 54 of the transposase.

24. A genetic construct as claimed in Claim 22 comprising a nucleotide sequence that encodes a proline residue at amino acid 372 of the transposase.

25. A genetic construct as claimed in Claim 22 comprising a nucleotide sequence that encodes a lysine residue at amino acid 54 of the transposase and a proline residue at amino acid 372 of the transposase.

26. A genetic construct as claimed in Claim 22 comprising the nucleotide sequence of SEQ ID NO:1.

27. A genetic construct comprising:  
a transposable DNA sequence flanked at its 5' and 3' ends by an 18 or 19 base pair flanking DNA sequence comprising nucleotide A at position 10, nucleotide T at position 11, and nucleotide A at position 12.

28. The construct of Claim 27 further comprising, at position 4 of the flanking sequence, a nucleotide selected from the group consisting of T or A.

29. The construct of Claim 27 further comprising, at position 15 of the flanking sequence, a nucleotide selected from the group consisting of G or C.

30. The construct of Claim 27 further comprising, at position 17 of the flanking sequence, a nucleotide selected from the group consisting of T or A.

31. The construct of Claim 27 further comprising, at position 18 of the flanking sequence, a nucleotide selected from the group consisting of G or C.

32. The construct as claimed in Claim 27 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACACATCT-3'.

33. The construct as claimed in Claim 27 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACAGATCT-3'.

34. A method for in vitro transposition, the method comprising the steps of:

combining a donor DNA molecule that comprises a transposable DNA sequence of interest, the DNA sequence of interest being flanked at its 5'- and 3'-ends by Tn5 outside end repeat sequences, with a target DNA molecule and a Tn5 transposase modified relative to wild type Tn5 transposase in a suitable reaction buffer at a temperature below a physiological temperature until the modified transposase binds to the outside end repeat sequences; and

raising the temperature to a physiological temperature for a period of time sufficient for the enzyme to catalyze in vitro transposition,

wherein the modified transposase comprises a change relative to the wild type Tn5 transposase that causes the modified transposase to bind to the Tn5 outside end repeat sequences with greater avidity than the wild type Tn5 transposase, and a change relative to the wild type Tn5 transposase that causes the modified transposase to be less likely than the wild type transposase to assume an inactive multimeric form.

35. A method as claimed in Claim 34 wherein the change that causes the modified transposase to bind with greater avidity is characterized as a substitution mutation at position 54 of the wild type transposase.

36. A method as claimed in Claim 35 wherein position 54 is a lysine.

37. A method as claimed in Claim 34 wherein the change that causes the modified transposase to be less likely to assume an inactive multimeric form is characterized as a substitution mutation at position 372 of the wild type transposase.

38. A method as claimed in Claim 37 wherein position 372 is a proline.

39. A method as claimed in Claim 34 wherein the modified transposase further comprises a substitution mutation at position 56 of the wild type transposase.

40. A method as claimed in Claim 39 wherein position 56 is an alanine.

41. A method as claimed in Claim 34 wherein the DNA sequence of interest is flanked at its 5'- and 3'-ends by an 18 or 19 base pair flanking DNA sequence comprising nucleotide A at position 10, nucleotide T at position 11, and nucleotide A at position 12.

42. The method as claimed in Claim 41 wherein the flanking sequence further comprises a nucleotide at position 4 selected from the group consisting of A or T.

43. The method as claimed in Claim 41 wherein the flanking sequence further comprises a nucleotide at position 15 selected from the group consisting of G or C.

44. The method as claimed in Claim 41 wherein the flanking sequence further comprises a nucleotide at position 17 selected from the group consisting of A or T.

45. The method as claimed in Claim 41 wherein the flanking sequence further comprises a nucleotide at position 18 selected from the group consisting of G or C.

46. The method as claimed in Claim 41 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACACATCT-3'.

47. The method as claimed in Claim 41 wherein the flanking sequence has the sequence 5'-CTGTCTCTTATACAGATCT-3'.

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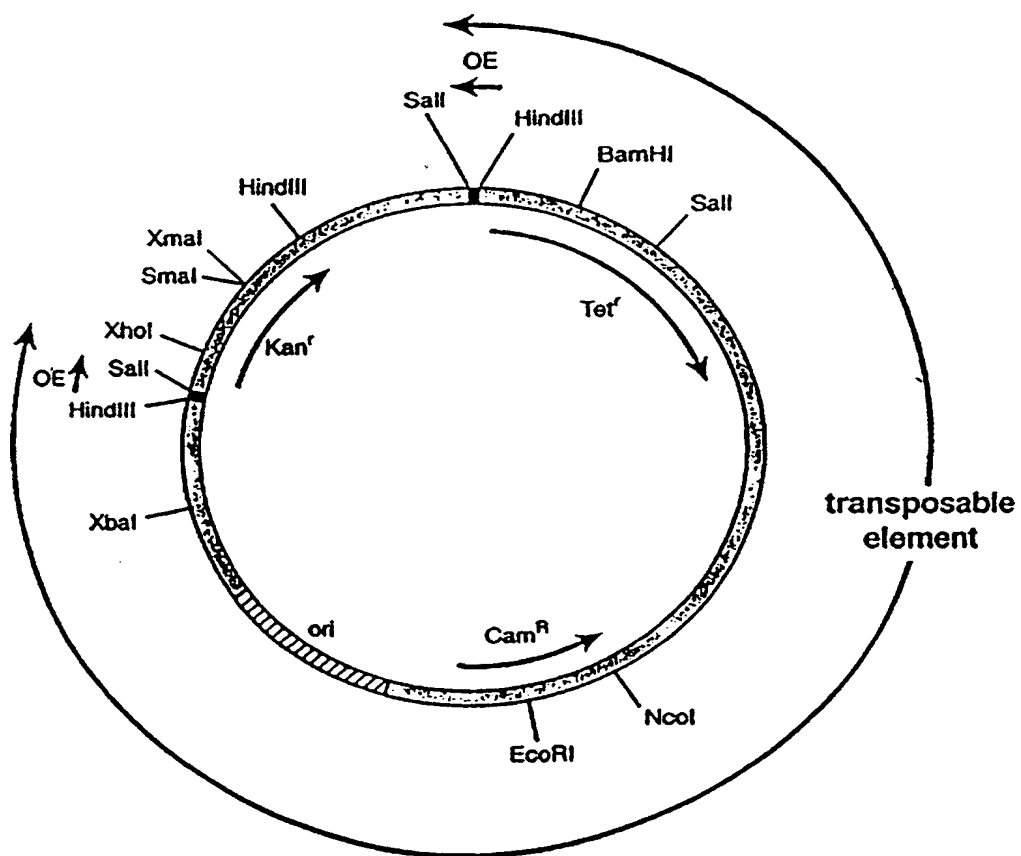
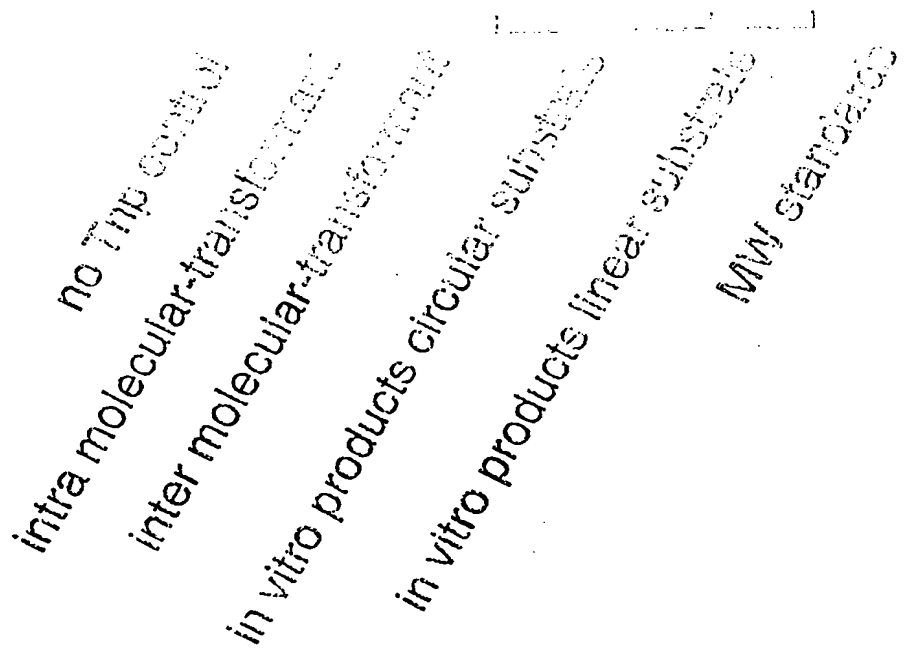
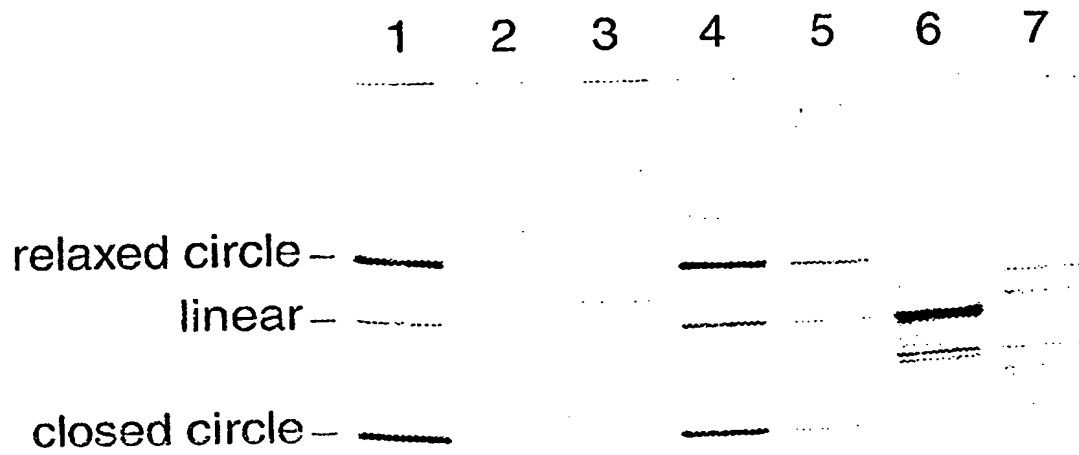
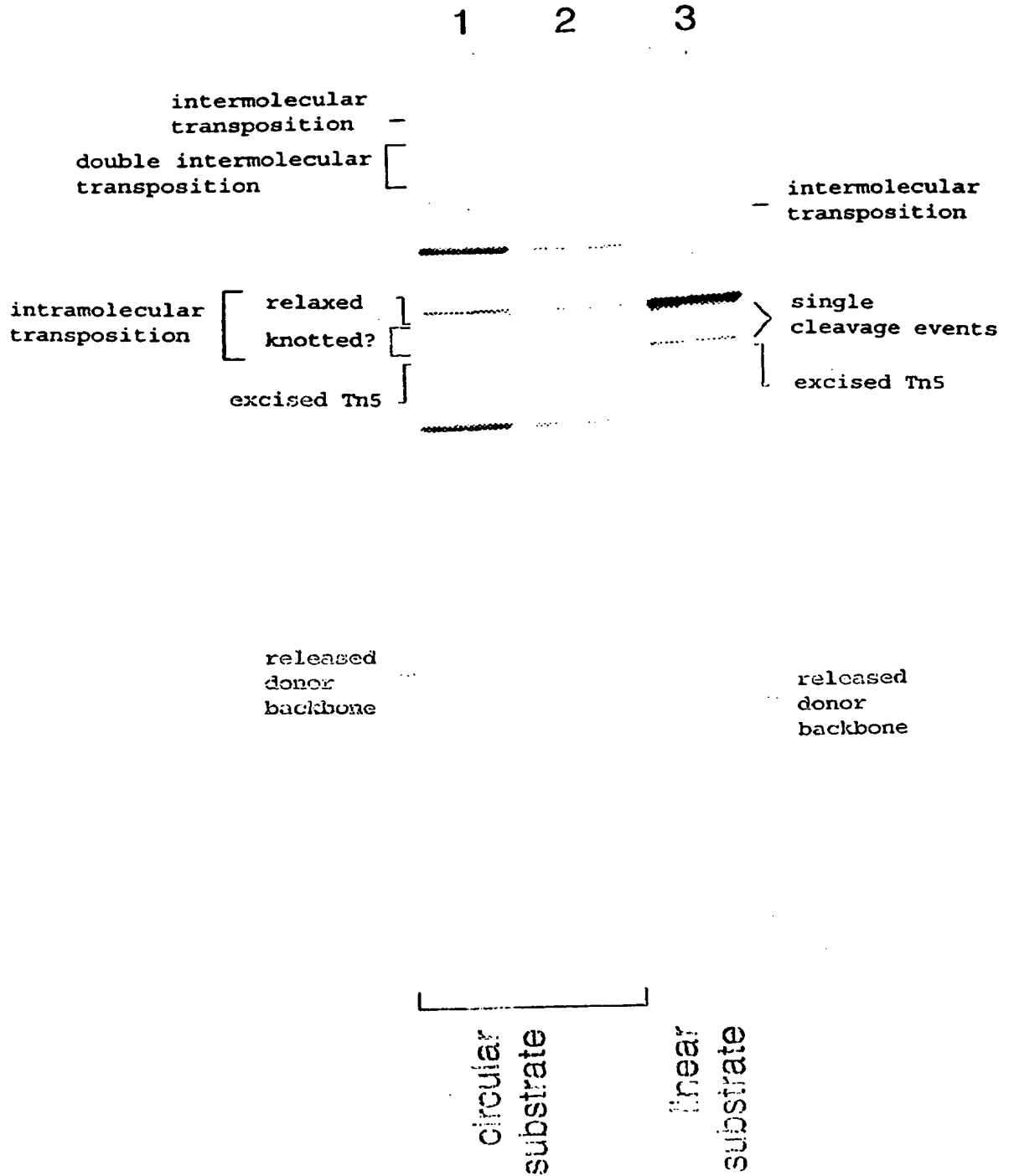


FIG 1



**FIG 2**  
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**FIG 3**  
**SUBSTITUTE SHEET (RULE 26)**

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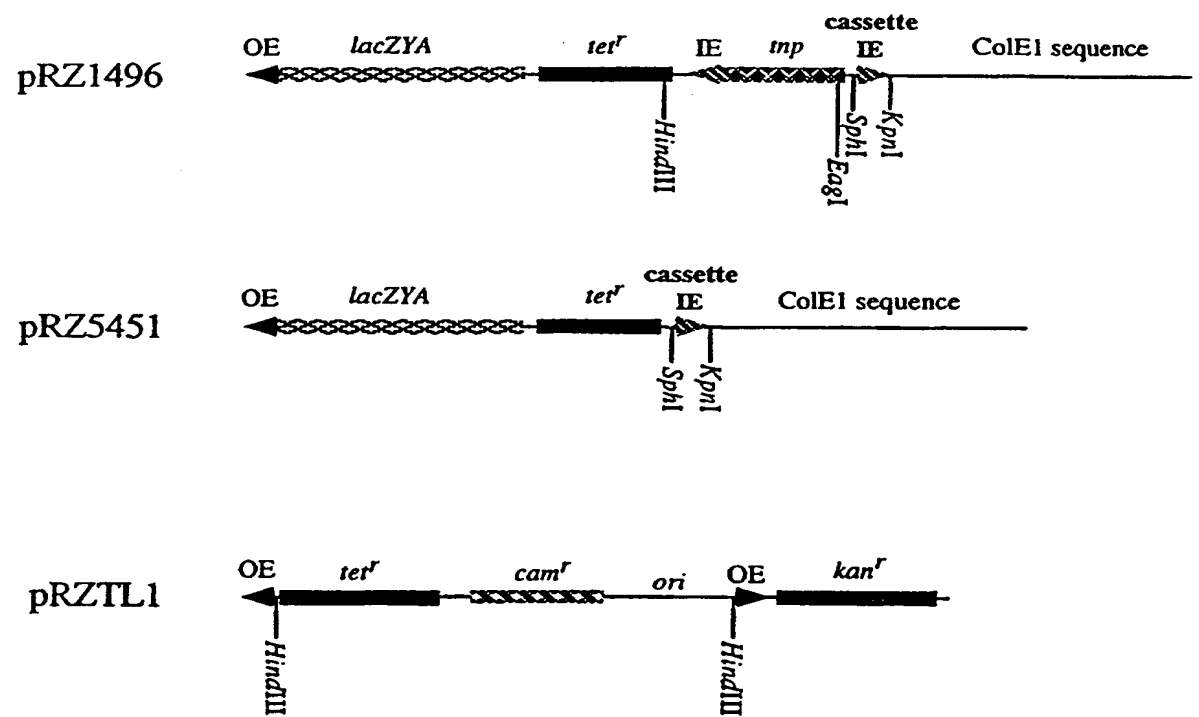


FIG 4

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a

## Papillation of IE Mutants with EK54 Tnp

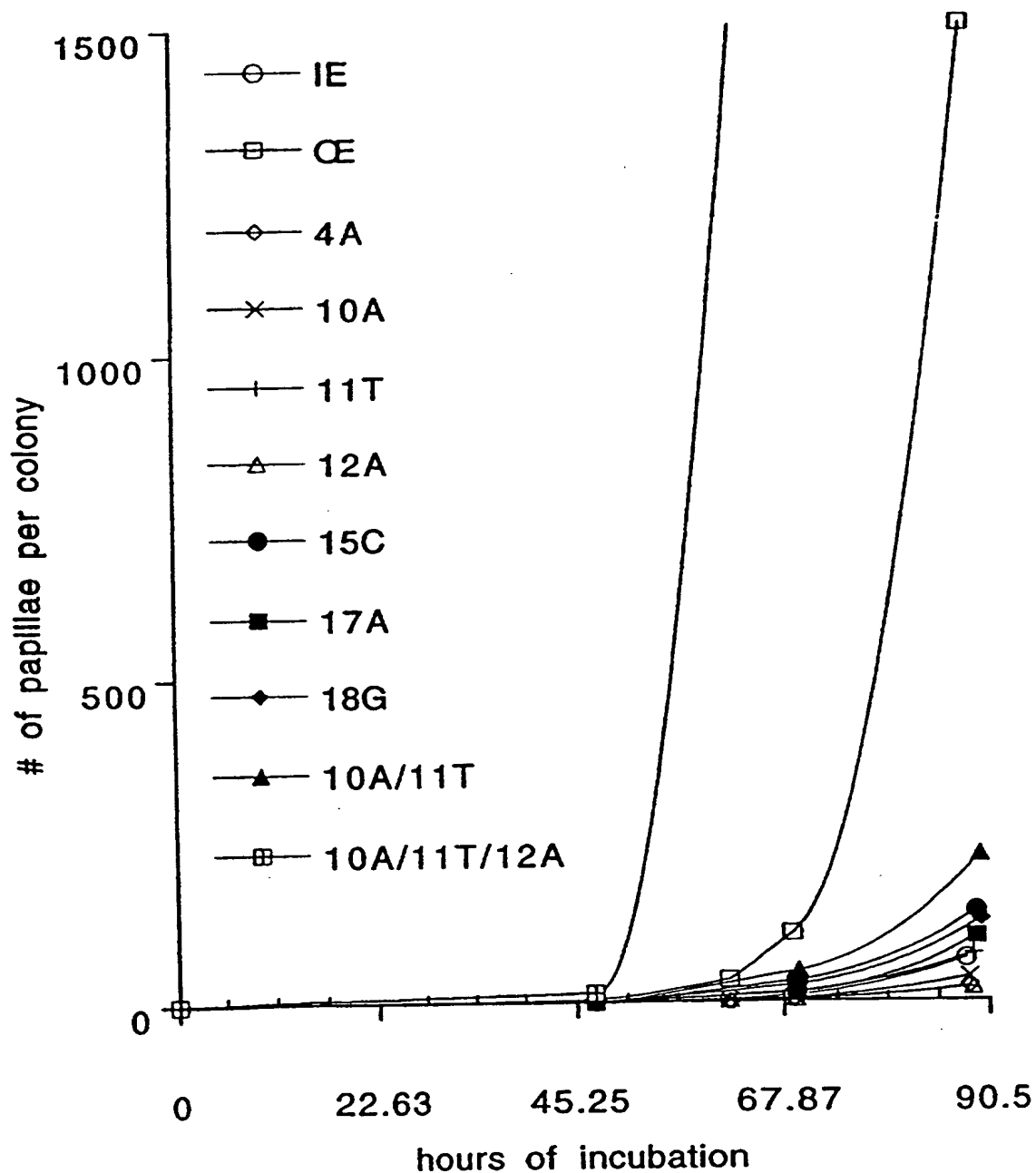
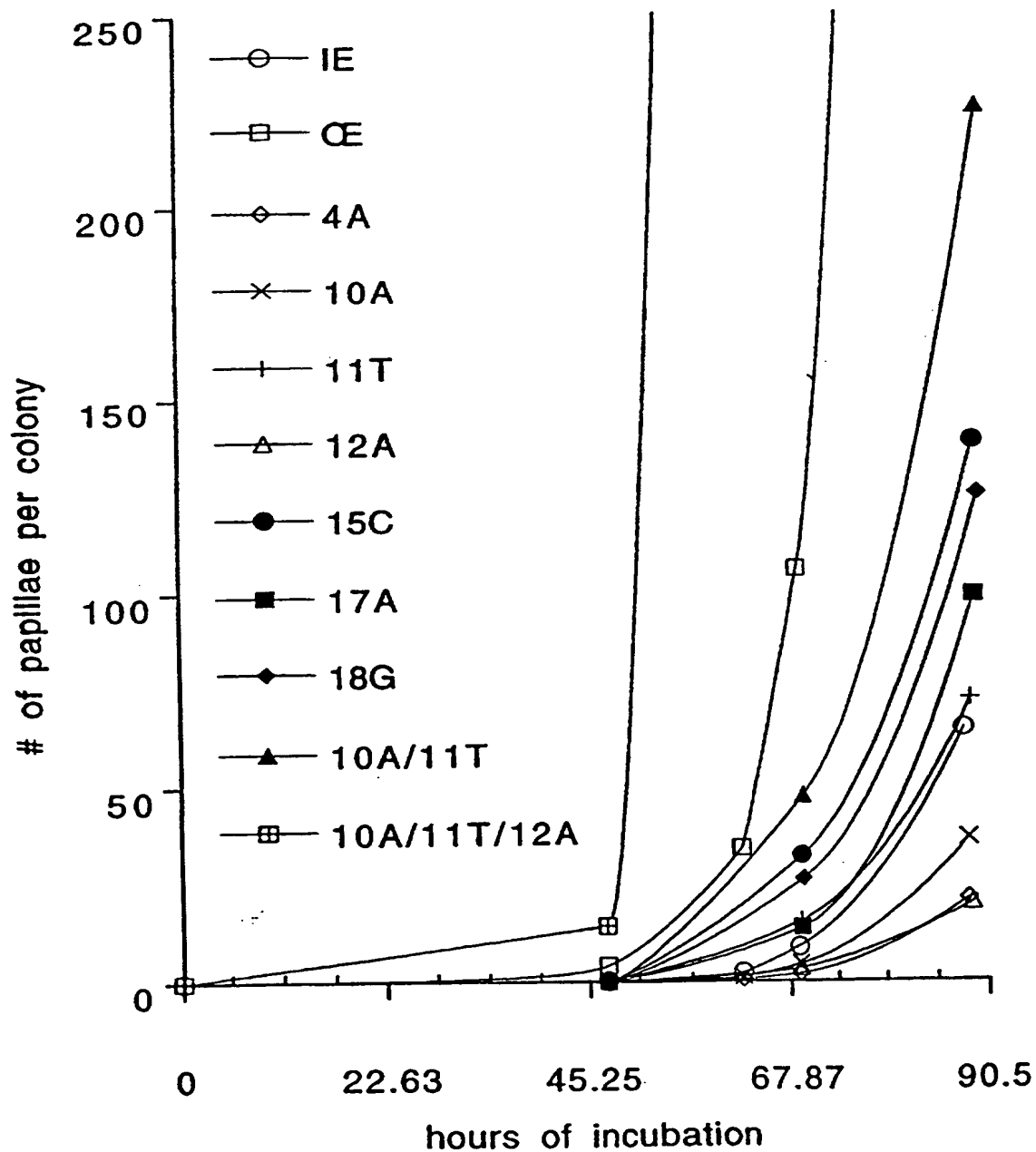
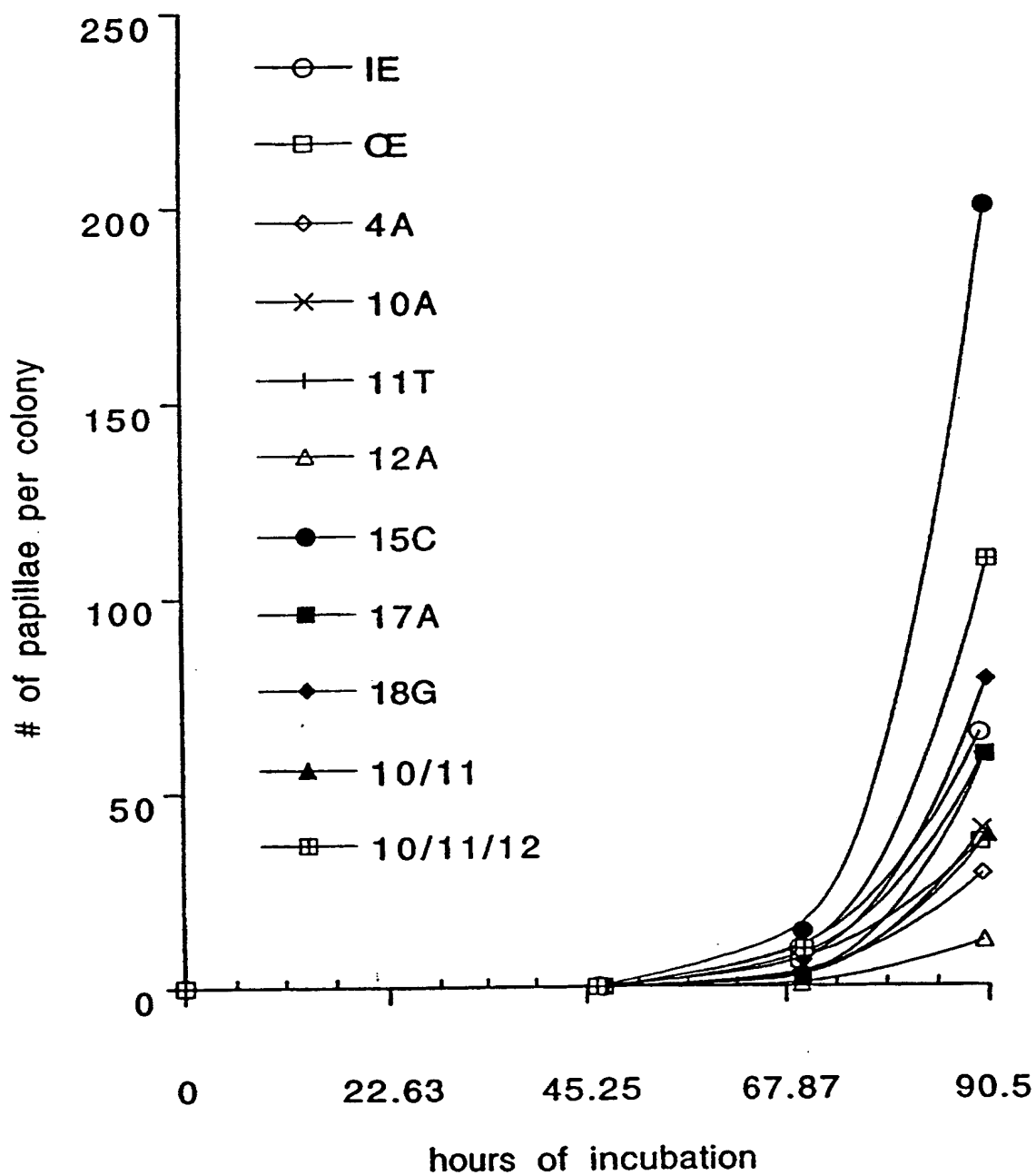


FIG 5

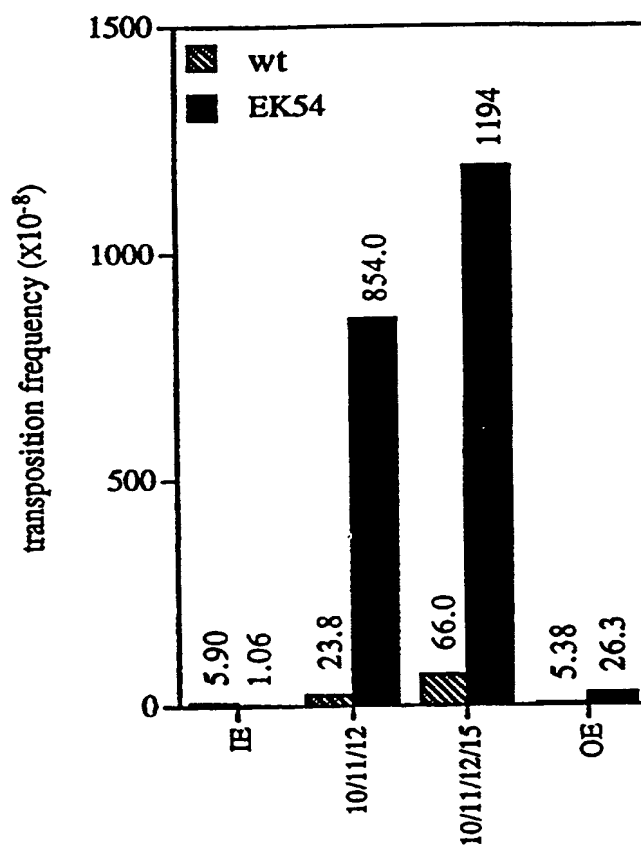
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**b****Papillation of IE Mutants with EK54 Tnp****FIG 6**

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**Papillation of IE Mutants with wt Tnp****FIG 7**

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**a****FIG 8**

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/15941

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/55 C12N9/22 C12N15/90 C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHOU M ET AL: "Three types of novel mutations in the NH-2-terminus of Tn5 transposase: Structure-function of transposase." KEYSTONE SYMPOSIUM ON TRANSPOSITION AND SITE-SPECIFIC RECOMBINATION: MECHANISM AND BIOLOGY, PARK CITY, UTAH, USA, JANUARY 21-28, 1994. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (18B). 1994. 45. ISSN: 0733-1959, XP002052633 see the whole document --- -/--	1-3, 15-17, 22, 23, 26, 34-36



Further documents are listed in the continuation of box C



Patent family members are listed in annex.

## \* Special categories of cited documents

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Date of the actual completion of the international search

20 January 1998

Date of mailing of the international search report

03/02/1998

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/15941

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEINREICH M D ET AL: "Evidence that the cis preference of the Tn5 transposase is caused by nonproductive multimerization." GENES & DEVELOPMENT 8 (19). 1994. 2363-2374. ISSN: 0890-9369, XP002052634 cited in the application see the whole document ---	1,4,5, 15,18, 19,22, 24,26, 34,37,38
Y	DELONG, ALISON ET AL: "Trans-acting transposase mutant from Tn5" PROC. NATL. ACAD. SCI. U. S. A. (1991), 88(14), 6072-6 CODEN: PNASA6;ISSN: 0027-8424, 1991, XP002052635 see the whole document ---	1-47
Y	WIEGAND, TORSTEN W. ET AL: "Characterization of two hypertransposing Tn5 mutants" J. BACTERIOL. (1992), 174(4), 1229-39 CODEN: JOBAAY;ISSN: 0021-9193, February 1992, XP002052636 see the whole document ---	1-47
Y	WIEGAND, TORSTEN WALTER: "Transposase mutants that increase the transposition frequency of Tn5" (1993) 164 PP. AVAIL.: UNIV. MICROFILMS INT., ORDER NO. DA9315014 FROM: DISS. ABSTR. INT. B 1993, 54(6), 2886, 1993, XP002052637 see the whole document ---	1-47
Y	WEINREICH M D ET AL: "A functional analysis of the Tn5 transposase. Identification of domains required for DNA binding and multimerization." J. MOL. BIOL., vol. 241, 1993, pages 166-177, XP002052638 see the whole document ---	1-47
Y	JILK R A ET AL: "The organization of the outside end of transposon Tn5." JOURNAL OF BACTERIOLOGY, vol. 178, no. 6, March 1996, pages 1671-1679, XP002052640 see the whole document ---	1-47
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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/15941

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R.C. JOHNSON ET AL.: "DNA sequences at the ends of transposon Tn5 required for transposition." NATURE, vol. 304, 21 July 1983, pages 280-282, XP002052641 cited in the application see the whole document ----	1-47
P,Y	YORK, DONA ET AL: "Purification and biochemical analyses of a monomeric form of Tn5 transposase" NUCLEIC ACIDS RES. (1996), 24(19), 3790-3796 CODEN: NARHAD;ISSN: 0305-1048, 1996, XP002052642 see the whole document ----	1-47
P,X	ZHOU M ET AL: "Tn5 transposase mutants that alter DNA binding specificity." JOURNAL OF MOLECULAR BIOLOGY 271 (3). 1997. 362-373. ISSN: 0022-2836, XP002052643 see the whole document ----	1-47
P,X	YORK, DONA ET AL: "DNA binding and phasing analyses of Tn5 transposase and a monomeric variant" NUCLEIC ACIDS RES. (1997), 25(11), 2153-2160 CODEN: NARHAD;ISSN: 0305-1048, 1997, XP002052645 see the whole document -----	1-47

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